

The *in vivo* production of *Heterorhabditis zealandica* and *Heterorhabditis bacteriophora*

by

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Declaration

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Abstract

The agricultural industry in South Africa is dominated by the use of insecticides. Producers rely heavily on chemicals that cause increased risk to health, the environment and ecology, rapid resistance development in key insect pests and pesticide residues on crops. The increased concern regarding the impact of these pest management practices on the environment and alternative pest management strategies are being investigated. Entomopathogenic nematodes (EPNs) have been identified as being promising biological control agents of key insect pests. The two EPN genera that have shown promise for use as biological control agents within an integrated pest management programme are *Steinernema* and *Heterorhabditis*. Commercialisation and the successful use of EPNs to control pests in North America, Australia, Europe and Asia have confirmed the effectiveness of these organisms as biological control agents. Unfortunately, EPNs in large enough numbers for commercial field applications are not yet available on the South African market. Large numbers of EPNs can be produced through either *in vivo* or *in vitro* culturing practices. The objective of this study was to streamline the *in vivo* production process by using two endemic EPN species, *Heterorhabditis zealandica* (SF41) and *H. bacteriophora* (SF351). These EPN isolates have been shown to be effective control agents of codling moth *Cydia pomonella*, false codling moth *Thaumatotibia leucotreta*, obscure mealybug *Pseudococcus viburni*, and the banded fruit weevil *Phlyctinus callosus*.

A comparative study was conducted to identify suitable host insects for EPN production of local *H. zealandica* (SF41) and *H. bacteriophora* (SF351) strains. Hosts were selected according to their susceptibility to the two EPN species used, their general availability and the ease and cost of rearing. Wax moth larvae *Galleria mellonella* (WML) and mealworms *Tenebrio molitor* (MW) were selected as hosts. In order to produce nematodes of consistent quality, a continuous source of host insects reared on a standardised diet was required. WML and MW were each reared on five different diets in the dark at $\pm 26^{\circ}\text{C}$. A superior diet for each host was selected according to the diet that produced, on average, the larvae with the highest body mass within a specific timeframe. The heaviest WML, at an average weight of 0.19 g per larva, were produced on a diet consisting of 118 g wheat flour, 206 g wheat bran, 118 g milk powder, 88 g brewer's yeast, 24 g wax powder, 175 ml honey and 175 ml glycerol. The heaviest MW larvae weighed, on average, 0.0154 g per larva, and were produced on a diet consisting of 100% wheat bran.

To confirm the hypothesis that a linear relationship exists between the weight of a host and the number of nematodes produced from that host, a study was conducted to determine the number of *H. zealandica* and *H. bacteriophora* produced per g of host. WML, MW, codling moth larvae and false codling moth larvae were weighed individually and inoculated with the two nematode species respectively. In addition, nematode production in frozen MW and WML was tested. The number of nematodes harvested from each host was counted, and the average number of nematode progeny produced in each host was calculated. A significant linear correlation between the weight of WML and MW and the number of *H. zealandica* and *H. bacteriophora* respectively produced confirmed the hypothesis that nematode production within the specified host increases with an increase in host weight. WML produced the highest number of *H. zealandica* and *H. bacteriophora* per g of host ($1\,459\,205 \pm 113\,670$ and $1\,898\,512 \pm 94\,355$), followed by MW larvae ($836\,690 \pm 121\,252$ and $414\,566 \pm 67\,017$). Lower numbers of *H. zealandica* and *H. bacteriophora* per g codling moth ($57\,582 \pm 10\,026$ and $39\,653 \pm 8\,276$) and per g false codling moth ($192\,867 \pm 13\,488$ and $97\,652 \pm 23\,404$) were produced.

Successful infection of a suitable insect host is one of the key factors in an efficient *in vivo* nematode production process. Three inoculation techniques were compared using *H. zealandica* and *H. bacteriophora*: inoculation with a pipette; shaking of hosts in the nematode inoculum; and immersion of hosts in the nematode suspension. With each inoculation technique, WML and MW were used as host larvae and were inoculated with nematodes at a concentration of 200 infective juveniles (IJs) / larva. The percentage mortality of insect hosts was determined after two days, and EPN infectivity, confirmed by colour change and dissection, after seven days. The highest percentage EPN infection was obtained using pipetting for both nematode isolates and hosts. Nematode infection rates for all nematode-host combinations obtained with pipetting were above 90%, with the exception of MW inoculation with *H. bacteriophora*, where the percentage of infection obtained was 76%. The current study conclusively demonstrated that variations in infection levels occur, depending on the inoculation technique used. In an additional effort to enhance infectivity during inoculation, *H. zealandica*, *H. bacteriophora* and MW were subjected to host-stressor regimes and to nematode-infectivity-enhancing additives. Three treatments, plus a control treatment, were compared. Exposing MW to 70°C tap water prior to inoculation did not increase infection levels. On the contrary, reduced infection levels were observed with host immersion in 70°C tap water followed by inoculation with *H. bacteriophora*, compared to the control. Only 12% infection was obtained compared to the 48% infection achieved in the control. Infection obtained using *H. zealandica* was 21%. Treating *H. zealandica* and *H. bacteriophora* IJs with $\text{Mn}^{2+}\text{SO}_4 \cdot \text{H}_2\text{O}$ in a suspension, prior to inoculating MW, did not significantly enhance nematode virulence. Inoculation of MW with treated *H. zealandica* IJs led to an infection rate of 81%, compared to the control, with which 80% infection rate was obtained. *Heterorhabditis bacteriophora* caused 47% MW infection, compared to the control, which was subject to 48% infection. A combination of the two

above-mentioned treatments did not enhance the infection levels either. Immersing MW into 70°C tap water prior to inoculation with nematodes treated with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ led to infection levels of 13% and 9% respectively when *H. bacteriophora* and *H. zealandica* were used. Future research is required to optimise the protocol used in this study of subjecting MW and local nematode isolates to stressor regimes.

The ability of two formulations to maintain biological activity and virulence of *H. zealandica* was investigated. A quality standard control measure was used to measure the percentage survival and virulence of formulated *H. zealandica* over a period of 21 days. IJs were formulated into Pesta granules and coconut fibres, while nematodes stored in tap water served as the control. The numbers of live *H. zealandica* in Pesta granules and coconut fibres decreased drastically after seven days of storage. The survival of nematodes in Pesta granules dropped to 9.79% after 21 days compared to the control, where the survival rate was 79.79%. Nematode survival in coconut fibres was even lower, at 25.84% after seven days and 2.25% after 21 days. After 21 days in storage, 100%+of nematodes survived in the control for coconut fibres. The application of the standard quality control measure, which was used to determine the virulence of formulated *H. zealandica*, proved to be ineffective. Higher MW mortality rates were obtained in the control where no nematodes were added to larvae, compared to where nematodes were added in varying dosages. However, adjusting certain aspects in the protocol of this quality control measure specifically to accommodate local conditions could possibly make it a more effective tool for measuring endemic nematode virulence.

Uittreksel

Die landboubedryf in Suid-Afrika word oorheers deur die gebruik van insekdoders. Vervaardigers steun swaar op chemikalieë wat toenemend gesondheids-, omgewings- en ekologiese risiko's, asook die snelle ontwikkeling van weerstand in sleutelinsekteplae veroorsaak, en wat reste van plaagdoders op gewasse laat. Na aanleiding van toenemende besorgdheid oor die impak van hierdie plaagbestuurspraktyke op die omgewing, word alternatiewe plaagbestuurstrategieë ondersoek. Entomopatogeniese nematodes (EPNs) is geïdentifiseer as belowende biologiese beheeragente van sleutelinsekteplae. Die twee EPN genera wat belofte inhou vir gebruik as biologiese beheeragente binne 'n geïntegreerde plaagbestuursprogram is *Steinernema* en *Heterorhabditis*. Kommersialisering en die geslaagde gebruik van EPNs om insekplae te beheer in Noord-Amerika, Australië, Europa en Asië, het die doeltreffendheid van hierdie organismes as biologiese beheeragente bevestig. Ongelukkig is EPNs in groot genoeg getalle vir kommersiële aanwending in die veld nog nie op die Suid-Afrikaanse mark beskikbaar nie. Groot getalle EPNs kan deur *in vivo* en *in vitro* teling verkry word. Die doelwit van hierdie studie was om die *in vivo* produksieproses te stroomlyn deur die gebruik van twee endemiese EPN spesies, *Heterorhabditis zealandica* (SF41) en *H. bacteriophora* (SF351). Hierdie EPN isolate is deur navorsing bewys om doeltreffende beheeragente van kodlingmot *Cydia pomonella*, vals kodlingmot *Thaumotobia leucotreta*, ligrooswitluis *Pseudococcus viburni*, en gebande vrugtekalanders *Phlyctinus callosus* te wees.

'n Vergelykende studie is gedoen om geskikte gasheerinsekte vir EPN produksie van plaaslike *H. zealandica* (SF41) en *H. bacteriophora* (SF351) isolate te vind. Gashere is geselekteer op grond van vatbaarheid vir die EPN spesie wat gebruik word, en algemene beskikbaarheid en gemak en koste van teling. Wasmotlarwes *Galleria mellonella* (WML) en meelwurms *Tenebrio molitor* (MW) is as gashere gekies. Ten einde nematodes van konsekwente kwaliteit te teel, word 'n deurlopende bron van gasheerinsekte benodig wat op 'n gestandaardiseerde dieet voed. WML en MW is onderskeidelik op vyf verskillende diëte geteel by $\pm 26^{\circ}\text{C}$ in die donker. Die beste dieet vir elke gasheer is gekies op grond van die dieet wat, gemiddeld, die swaarste larwes binne 'n spesifieke tydsraamwerk opgelewer het. Die swaarste WML, teen 'n gemiddelde massa van 0.19 g per larwe, is geteel op 'n dieet wat bestaan het uit 118 g koringmeel, 206 g semels, 118 g melkpoeier, 88 g brouersgis, 24 g verpoeierde was, 175 ml heuning en 175 ml gliserol. Die swaarste MW larwes het gemiddeld 0.0154 g per larwe geweeg en is geteel op 'n dieet van 100% semels.

Ten einde die hipotese te bevestig dat 'n lineêre verwantskap bestaan tussen die massa van 'n insekgasheer en die aantal nematodes wat deur daardie gasheer geproduseer word, is 'n studie gedoen om die aantal *H. zealandica* en

H. bacteriophora per gasheergram te bepaal. WML, MW, kodlingmotlarwes en vals kodlingmotlarwes is individueel geweeg en met infektiewe larwes van die twee onderskeidelike EPN spesies geïnkuleer. Daarbenewens is die vermeerdering van nematodes in bevrore MW en WML ook getoets. Die aantal nematodes wat in elke gasheer geoes is, is getel, en die gemiddelde nematode-afstammeling in elke gasheer bereken. 'n Beduidende lineêre korrelasie tussen die massa van WML en MW en die aantal *H. zealandica* en *H. bacteriophora* wat onderskeidelik geproduseer is, het die hipotese bevestig dat nematode-vermeerdering binne hierdie gashere toeneem namate die gasheermassa toeneem. WML het die meeste *H. zealandica* en *H. bacteriophora* per gasheergram opgelewer ($1\,459\,205 \pm 113\,670$ en $1\,898\,512 \pm 94\,355$ onderskeidelik), gevolg deur MW larwes ($836\,690 \pm 121\,252$ en $414\,566 \pm 67\,017$ onderskeidelik). Laer getalle *H. zealandica* and *H. bacteriophora* per gram kodlingmot ($57\,582 \pm 10\,026$ en $39\,653 \pm 8\,276$) en per gram vals kodlingmot ($192\,867 \pm 13\,488$ en $97\,652 \pm 23\,404$) is egter geproduseer.

Een van die sleutelfaktore vir die doeltreffendheid van die *in vivo* vermeerdering van nematodes is geslaagde gasheerinfeksie. Drie inokulasietegnieke is dus geëvalueer en vergelyk deur *H. zealandica* en *H. bacteriophora* te gebruik: inokulasie met 'n pipet, skud van gashere in 'n nematode-inokulum, en gasheerindompeling in 'n nematode-suspensie. WML en MW is as gashere gebruik vir elke inokulasietegniek, en is geïnkuleer met nematodes wat uit 'n konsentrasie van 200 infektiewe larwes (ILs) / insek larwe bestaan het. Die persentasie dooie insekgashere is na twee dae bepaal, en infeksie soos bevestig deur kleurverandering en disseksie, na sewe dae. Die hoogste persentasie infeksie deur sowel nematode-isolate as gashere te gebruik, was met die pipet-tegniek. Die infeksiekoerse vir alle nematode-gasheerkombinasies met die pipet-tegniek was hoër as 90%, met die uitsondering van MW-inokulasie met *H. bacteriophora*, waar die infeksie 76% was. Hierdie studie toon dat afwykings voorkom in die mate van gasheerinfeksie, na gelang van die inokulasietegniek wat gebruik is. In 'n bykomende poging om infeksie na inokulasie te verhoog, is *H. zealandica*, *H. bacteriophora* en MW onderwerp aan stressors en bymiddels om nematode-infeksie te bevorder. Drie behandelings, asook 'n kontrole-behandeling, is vergelyk. Infeksievlakke het nie verhoog deur MW voor inokulasie aan kraanwater van 70°C bloot te stel nie. Intendeel, laer infeksievlakke is opgemerk waar gashere in kraanwater van 70°C gedompel is en daarna met *H. bacteriophora* geïnkuleer is, vergelyke met die kontrole. Gasheerinfeksie van slegs 12% is verkry, vergelyke met 48% in die kontrole. Infeksie van 21% is met *H. zealandica* verkry. Die virulensie van nematodes het nie beduidend toegeneem deur *H. zealandica* en *H. bacteriophora* IL in 'n suspensie met $\text{Mn}^{2+}\text{SO}_4\text{H}_2\text{O}$ te behandel voor MW geïnkuleer is nie. Inokulasie van MW met behandelde *H. zealandica* IL het tot 'n infeksie van 81% gelei, vergelyke met die kontrole waar 'n infeksie van 80% behaal is. *H. bacteriophora* het 'n MW-infeksie van 47% veroorsaak, vergelyke met die kontrole se infeksiekoerse van 48%. 'n Kombinasie van die twee bogenoemde behandelings het eweneens nie gasheerinfeksievlakke verhoog nie. Die indompeling van meelwurms in kraanwater van 70°C voor inokulasie met nematodes wat met $\text{Mn}^{2+}\text{SO}_4\text{H}_2\text{O}$ behandel is, het tot gasheerinfeksie van 13% en 9%

onderskeidelik gelei wanneer *H. bacteriophora* en *H. zealandica* gebruik is. Toekomstige navorsing is nodig om die protokol te verbeter wat in hierdie studie gebruik is om MW en plaaslike nematode-isolate aan stressors te onderwerp.

'n Ondersoek is gedoen na die vermoë van twee formulasies om biologiese aktiwiteit en virulensie van *H. zealandica* te onderhou. 'n Kwaliteitsstandaardtegniek is gebruik om weekliks die persentasie oorlewing en virulensie van geformuleerde *H. zealandica* oor 'n tydperk van 21 dae te meet. IL is in Pesta korrels en klappervesel geformuleer, terwyl nematodes in kraanwater gedien het as kontrole. Die aantal lewende *H. zealandica* in Pesta korrels en klappervesel het drasties verminder na sewe dae in die formulasie. Oorlewing van nematodes in Pesta korrels het gedaal tot 9.79% na 21 dae vergelyke met die kontrole, waar 79.79% oorleef het. Nog minder nematodes - 25.84% - het na sewe dae in die klappervesel oorleef, en slegs 2.25% na 21 dae. Na 21 dae van berging het 100%+ van nematodes oorleef in die kontrole vir klappervesel. Die toepassing van die kwaliteitsstandaardtegniek om die virulensie van geformuleerde *H. zealandica* te bepaal, het ondoeltreffend geblyk. Verhoogde MW sterftesyfers is verkry in die kontrole waar geen nematodes by die inseklarwes gevoeg is nie, vergelyke met die byvoeging van hoër dosisse nematodes. Nietemin, die aanpassing van sekere aspekte in die protokol van hierdie kwaliteitsbeheermeting om spesifiek plaaslike toestande in ag te neem, sou dit moontlik 'n meer doeltreffende middel kon maak om die virulensie van endemiese nematodes te bepaal.

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Chapter 1

Literature review

In order to place the multifaceted topic of *in vivo* production of entomopathogenic nematodes (EPNs) into context, the literature review consists of three main sections. The first will focus on the role of nematodes as biological control agents: their distribution, biology and life cycle, their physical and behavioural characteristics, and their host range. The second discusses the history of nematode mass culture. Lastly, *in vivo* culturing, using live insect hosts and factors that influence their efficacy and production, are discussed.

The role of entomopathogenic nematodes as biological control agents

Annually insect pests cause serious damage to agricultural crops, which leads to substantial financial losses in agriculture worldwide (Wyniger 1962; Oerke 2006). In order to control these pests, humans have relied heavily on synthetic chemical pesticides. Although chemical pesticides have played an important role in controlling agriculturally important pests, their use has also led to pesticide resistance, secondary pest outbreaks, pesticide residues on crops, and health risks to animals and humans. Pesticide availability is becoming increasingly restricted as a result of more stringent safety requirements, which have led to many products being banned (Moazami 2002). These negative consequences, and the current status of synthetic compounds, have contributed to an increased interest in natural approaches. Such approaches are based on the development and incorporation of more environmentally benign alternatives in pest management practices. Biological control is one such alternative.

Biological control is defined as the management of a pest by the deliberate use of living organisms (natural or applied) to maintain the pest population density at a lower level than would occur in the absence of the organism (DeBach 1964). Natural biological control happens where co-evolved natural enemies suppress pest populations without human intervention, while applied biological control implies human intervention to enhance activities by natural enemies. Applied biological control can be split up into three different categories: classical, inundative (or augmentative) and conservational biological control (Vincent et al. 2007). EPNs from the genera *Steinernema* and *Heterorhabditis* have

been identified as promising biological control agents for inundative biological control of a vast array of agriculturally important insect pests (Ehlers 1996; Hazir et al. 2003).

EPNs are naturally occurring, non-segmented, colourless, elongated, insect-parasitic roundworms with lengths ranging from 0.4 mm to 1.1 mm. The nematodes live in a variety of soil types and are able to infect over 200 insect hosts under laboratory conditions (Hazir et al. 2003). Apart from EPNs being especially efficacious against soil-borne pests, they also show great potential to suppress pests above ground via foliar application (Kaya et al. 1984; Nachtigall and Dickler 1992; Unruh and Lacey 2001; Arthurs et al. 2004; Shapiro-Ilan et al. 2006). EPNs occur naturally in the soil environment where they perform optimally against soil-borne insect pests. The nematodes have the ability to immediately suppress population levels of their insect hosts within one to two days after infection. They also have the ability to persisting two to three weeks in the field (Gaugler 1988; Kaya et al. 1993; Burnell and Stock 2000). As a component of a pest management programme, EPNs can be periodically introduced to maintain host population levels below what they would have been in the absence of the nematodes.

Nematodes in the soil can generally be divided into two groups: beneficial (which include EPNs) and non-beneficial (plant-parasitic) nematodes (Kaya et al. 1993; Bird and Bird 2001). EPNs are beneficial nematodes, because of their ability to parasitise insects without harm to humans, animals or the environment. For the biological control of insects, major focus has been on the two nematode genera, *Heterorhabditis* and *Steinernema*. *Steinernema* and *Heterorhabditis* are characterised by their respective highly virulent associated symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, which are directly responsible for the death of insect hosts (Moazami 2002). Characteristics that enhance the attractiveness of nematodes for use as pest control agents are: their ability to seek out a host and to kill it within 48 h (Poinar 1972); their lack of impact on non-target organisms; the relatively easy and economically feasible mass artificial propagation of EPNs; their definite possibility of incorporation into integrated pest management programmes (Grewal 2002); their compatibility with many chemical pesticides; their ability to be applied by means of standard spraying equipment and through drip irrigation lines where pressure does not exceed 2000 kPa (Georgis 1990); the lack of health, environmental or ecological risks once they are applied in the field; that they are self-sustaining organisms at optimal conditions; and, in some cases, their better performance compared to chemical control measures (Ehlers 2003). Disadvantages of EPNs include their sensitivity to UV light, low moisture conditions and extreme temperatures.

EPNs have been commercialised and are successfully used on a commercial scale for pest control in North America, Europe, Japan, China and Australia (Ehlers and Hokkanen 1996). Many other countries are conducting research on the development of EPNs as biological control agents (Kaya et al. 2006). High-value cropping systems have been successfully protected from key pests by inundative use of nematodes, both through soil and foliar applications

(Ehlers 1996). Some of these pests include citrus root weevils in citrus; black vine weevils in nurseries; mole crickets on turf grass; peach borer and codling moth on apples; and black cutworms (Klein 1990; Georgis and Hague 1991; Kaya and Gaugler 1993). In South Africa, research on the use of EPNs has mainly been done on the control of codling moth (De Waal et al. 2008, 2010, 2011); mealybug (Stokwe 2009); the banded fruit weevil (Ferreira 2010); and false codling moth (Malan et al. 2011).

Even though indigenous EPNs have proven to be effective against key insect pests under laboratory conditions in South Africa, nematodes are not yet available on the South African market as commercially formulated biological pest control products. Unlike most European countries, Northern America and the United Kingdom, where nematodes are exempted from registration, the importation of exotic EPNs and the application of these nematodes as biological control agents into South Africa is subject to regulation by the Department of Agriculture, Forestry and Fisheries (DAFF). It will soon also be subjected to regulation by the Department of Environmental Affairs (DEA). The South African Agricultural Pests Act 36 of 1983 prohibits importation of exotic organisms, including foreign EPNs, without a permit and a full-impact study (Klein et al. 2011). Therefore, several surveys have been conducted in South Africa with the aim of finding and identifying suitable local EPNs that could be used as biological control agents (Malan et al. 2006, 2008; Hatting et al. 2009; Malan et al. 2011). Besides the restrictions placed on the importation of EPNs delaying adoption of these biological control agents in the field, exotic strains may have negative effects on non-target organisms and lead to biological pollution through the reduction of endemic populations of EPNs (Ehlers 2005). Endemic strains are also climatically better adapted to local ecological conditions and could, consequently, perform more efficiently compared to exotic strains. Isolates of *Heterorhabditis bacteriophora* Poinar, 1976 and *Heterorhabditis zealandica* Poinar, 1990 have been collected in surveys conducted in South Africa and have been identified as being very effective against key insect pests of deciduous and citrus fruits (De Waal et al. 2010; Malan et al. 2011). Therefore, the current study focuses on the EPN species concerned.

Distribution

EPNs are ubiquitous in cultivated and uncultivated soils throughout the world (Hominick et al. 1996; Hominick 2002; Stuart et al. 2006). *Heterorhabditis* and *Steinernema* nematodes are present on all continents, except Antarctica (Popiel and Hominick 1992). The occurrence of *Heterorhabditis* is often linked to tropical regions (Nguyen and Hunt 2007), but studies by Griffin et al. (1991) and Hominick et al. (1995) record the presence of heterorhabditids in semi-arid climate zones as well. *Heterorhabditis bacteriophora* was originally found in Brecon, South Australia, by Poinar in

1976 and occurs in regions of continental and Mediterranean climate in both the northern and southern hemispheres (Hominick et al. 1996). *Heterorhabditis zealandica* was originally reported from Auckland, New Zealand, in 1990 and has been isolated in New Zealand, Tasmania (Poinar 1990) and South Africa (Malan et al. 2006, 2011).

The first record of EPNs in South Africa was from Grahamstown, Eastern Cape in 1953, when individuals of this nematode group were found in the maize beetle, *Heteronychus arator* (Fabricius) (Harrington 1953). Surveys have since been conducted in the provinces of KwaZulu-Natal, the Eastern Cape, the Western Cape, the Free State, Gauteng and Mpumalanga (Spaull 1988, 1990, 1991; Malan et al. 2006, 2008; Hatting et al. 2009). In local surveys, several *Steinernema* and *Heterorhabditis* species were found. Unfortunately, the most common commercially produced species, *Steinernema carpocapsae* Weiser, 1955 and *Steinernema feltiae* Filipjev, 1934, have not yet been reported for South Africa (Kaya et al. 2006). However, new species described for South Africa include *Steinernema khoisanae* (Nguyen et al. 2006), *Heterorhabditis safricana* (Malan et al. 2006) and *Steinernema citrae* (Malan et al. 2011; Stokwe et al. 2011).

Biology and life cycle

The word ‘entomo’ means insect, and ‘pathogenic’ means to cause a disease, hence the term ‘entomopathogenic’ translates as disease-causing to insects. The free-living, non-feeding J3 infective juvenile (IJ), which is the invasive stage in the life cycle of EPNs, is used for insect control purposes. The body length of an IJ can range between 418 µm to 1 283 µm (Nguyen and Hunt 2007). The only function of the IJ is to locate and infect new insect hosts. The IJ, or dauer juvenile, is formed as a response to depleting food sources and harsh environmental conditions (Ehlers 2007), and is adapted to long-term survival in the soil. IJs actively move through the soil to seek their host through well-developed chemo-reception, and by means of tracing insect movement through extensive mechano-reception (Riga 2004). Once the IJ comes into contact with the host, it enters the insect through natural openings (mouth, anus, spiracles) or the body wall, which could be the case with *Heterorhabditis* species. The IJ of this genus possesses a dorsal tooth that facilitates penetration through intersegmental membranes by abrasion (Popiel and Hominick 1992).

A distinctive characteristic of EPNs is the presence of gram-negative symbiotic bacteria cells contained in special vesicles of *Steinernema* and in the intestine of *Heterorhabditis* species (Poinar and Thomas 1966; Bird and Akhurst 1983). *Xenorhabdus* and *Photorhabdus* are the symbiotic bacterial associates of *Steinernema* and *Heterorhabditis* respectively. A mutualistic relationship exists between the nematode and its associated bacteria: the nematode facilitates transport of the bacteria through the soil to the haemolymph of an insect, protecting it from the competitive soil

environment. In turn, the nematode relies on the pathogenic potential of the bacteria to kill the insect host, to supply the nutrient base once inside the host, and to suppress cadaver contamination by micro-organisms (Gaugler 2002).

After penetration of the insect host, the IJ releases bacterial cells in the haemocoel, and rapid multiplication of the cells takes place, which initiates nematode development. Toxins, antimicrobial agents and exo-enzymes produced by the bacteria cause death of the host soon thereafter by septicaemia (within approximately 48 h after nematode penetration) and preserve the cadaver from putrefaction. The nematodes feed on the bacteria cells and metabolised host tissue, developing and reproducing within the insect cadaver whilst nutrients are abundant. IJs develop to the fourth- stage juvenile (J4), after which *Heterorhabditis* develops into first-generation adult hermaphrodites and second- generation amphimictic adults (Kaya and Gaugler 1993), whilst *Steinernema* develops into amphimictic first- and second- generation adults (Poinar 1990). Completing one life cycle inside a host takes three to seven days for both *Steinernema* and *Heterorhabditis*. Depending on nutrient availability, one to three generations can be completed in an insect cadaver. Emergence from the cadaver commences 12-14 days post-penetration (Kaya and Koppenhöfer 1999), or as soon as nutrients become depleted. At this point, late-second-stage juveniles (J2) develop into infective juvenile stages and exit the cadaver in search of a new host (Gaugler 2002).

Physical and behavioural characteristics

Nematode behaviour is complex and can be voluntary, or influenced and changed through external physical and chemical stimuli (Gaugler and Bilgrami 2004). EPNs exhibit five types of voluntary movement and foraging behaviour: cruising; ambushing; a combination of cruising and ambushing; nictating; and jumping (Campbell and Kaya 2002; Burr and Robinson 2004). The behavioural characteristics of *S. carpocapsae* have been the most studied, whilst limited information is available on other species like *H. zealandica*. *Heterorhabditis bacteriophora* are known for their cruising behaviour, whereby they locate hosts by detecting volatile cues released by the host, and following the gradient leading them towards the host. Cruising nematodes are highly mobile and effective against stationary or slow-moving insect pests in the soil. If an IJ responds to volatile cues from a host, the nematode concerned can be identified as a cruiser (Lewis et al. 1993; Grewal et al. 1994a). Ambusher nematodes, in contrast, are far less mobile and exhibit the behavioural mechanism of nictating to attach to a passing host (Burr and Robinson 2004). Ambusher nematode strategy involves attaching to a bypassing host whilst remaining stationary at, or near, the soil surface (Campbell et al. 1996). Nictating behaviour involves the nematode lifting its body from the substrate and waving in loops while standing on its tail (Campbell and Kaya 1999).

Heterorhabditis zealandica and *S. carpocapsae* are examples of ambusher nematodes and are effective at finding sedentary, spatially patchy insect hosts (Campbell and Gaugler 1993). *Steinernema feltiae* is one example that shares characteristics of both ambushers and cruisers on the behavioural continuum. The species is able to attack both sedentary and mobile insects using the intermediate strategy. Jumping behaviour, which is exhibited by *Steinernema scapterisci* Nguyen & Smart, 1990, assists the nematode in attaching to a passing host, which is called external phoresis. External phoresis is also a common strategy used by more stationary-type nematodes to disperse (Downes and Griffin 1996). Jumping behaviour has not yet been reported in *Heterorhabditis* (Campbell and Kaya 2002). *Heterorhabditis* has a tendency to disperse actively downwards and is thus more effective against pests that occur relatively deep in the soil (Georgis and Hom 1992), compared to *Steinernema* that mostly disperses horizontally in the upper soil layers and which is, thus, better adapted to attacking insect pests feeding on the soil surface (Lewis et al. 1992, 1993; Campbell and Gaugler 1993).

As far as commercial nematode application is concerned, foraging strategies influence host specificity, application technique, nematode occurrence at certain soil depths, and the shelf-life period of nematode formulations (Downes and Griffin 1996). For example, *H. bacteriophora* has a higher metabolic rate compared to *S. feltiae*, because of its more active searching behaviour. Furthermore, IJs of *H. bacteriophora* are smaller in size (588 µm) compared to *S. feltiae* (879 µm) and thus have less energy reserve available compared to *S. feltiae*, which leads to the lower life expectancy of *H. bacteriophora*, and, in addition, shortens the shelf-life of formulated products containing *H. bacteriophora* (Lewis et al. 1995). Apart from voluntary behaviour and movement, sensory organs of nematodes cause them to react and to behave in a certain manner as a response to external stimuli, which could include chemical, mechanical, photo, thermo, electric and magnetic stimuli. The behaviour may also involve repulsion (moving away from toxic chemicals) or attraction (moving towards more suitable temperature); inter- and intraspecific reactions between species; coiling behaviour, as a response to sex pheromone release; or aggregation behaviour, as a response to desiccation, which is a characteristic survival technique (Croll 1970; Gaugler and Bilgrami 2004). Other nematode survival behaviour tactics include syncing their own life cycle with that of the host through developmental arrest (IJ stage); surviving dry conditions by partial anhydrobiosis, upon which metabolism is slowed; or coiling, which leads to less body surface being exposed to ambient environment and reduced water loss rate. Some research, however, states that Steinernematid and Heterorhabditid nematodes are exclusively quiescent anhydrobiotes, thus precluding the ability of nematodes to enter anhydrobiosis (Womersley 1990a, b; Glazer 2002).

Host range

The use of wax moth larvae, *Galleria mellonella* (L.), in surveys as bait to obtain nematodes from soil has led to limited information being available on the natural host range of EPNs. Such nematodes have a preferred host range and are not equally efficient at infecting all insects (Popiel and Hominick 1992). Even though it has been stated that EPNs have a broad host range, being able to infect about 200 different insect species (Ehlers 1996), these results were obtained in the laboratory, where optimal conditions exist for laboratory-cultured nematodes, and where the possibility of host adaptation exists (Peters 1996). Even though the natural host range of a considerable number of nematode species has been determined, only a few species have successfully been produced, marketed and used as biological insecticides (Georgis et al. 2006). A comprehensive review of natural insect hosts for *Heterorhabditis* and *Steinernema* was compiled by Peters (1996).

To obtain optimal pest suppression results, selection of the best suitable nematode for the target pest is of cardinal importance. Virulence towards the host and host-seeking behaviour are important factors to take into consideration when selecting EPN species as biocontrol agents against a specific pest (Lewis et al. 1992; Lewis 2002).

Factors that influence nematode efficacy and production

Extreme temperatures

Optimal temperatures for efficient functioning of EPNs vary among species (Grewal et al. 1994b). Some species may be better adapted to cold ambient temperatures, such as *S. feltiae*, which is a species that is able to perform optimally at temperatures below 15°C (Kung 1990; Kung et al. 1991; Grewal et al. 1994b; Berry et al. 1997; Shapiro and McCoy 2000; Hazir et al. 2001). *Steinernema riobrave* Cabanillas, Poinar & Raulston, 1994, in contrast, are more heat tolerant and remain efficient at temperatures of 29°C and above. *Heterorhabditis bacteriophora* can survive at -19°C for short intervals, as it is freeze tolerant. The sheath of *H. zealandica* can protect the nematode at temperatures as low as -32°C from freezing. The mean temperature at which optimal infectivity is achieved for *Heterorhabditis* is 25°C (Mason and Hominick 1995).

Extreme ambient temperatures can lead to desiccation, increased metabolic rate and, consequently, more rapid use of energy reserves, which leads to shorter generation times, decreased infectivity and lowered virulence (Kaya 1990; Grewal et al. 1994b). Although soil serves as a buffer to highly varied temperature changes, temperature can still have a

major influence on nematode efficacy in the soil and above ground during foliar application. Therefore, a means to lower the impact of temperature should be enforced, like choosing the right nematode strain, applying nematodes at the right time of the year, or postponing application until temperatures are optimal for the specific nematode used.

Moisture

Sufficient soil moisture is essential for nematode movement, persistence and pathogenicity in the field (Georgis and Gaugler 1991), and can be achieved if the area of application is irrigated before and after application (Womersley 1990a, b). However, suboptimal moisture levels can be beneficial when formulating nematodes. Water activity in the formulation can gradually be reduced to a point where the nematode enters a state of partial anhydrobiosis. During this stage, the nematode's metabolism ceases, which consequently leads to a lowered use of energy reserves, increasing the life span of the nematode under suboptimal moisture levels. Pre-application of water rehydrates formulated nematodes and they resume function as virulent individuals, ready to be applied in the field (Georgis and Kaya 1998).

Oxygen supply

As nematodes are aerobic organisms, a shortage of oxygen invariably leads to death (Kung 1990; Lewis and Perez 2004). Suboptimal soil types with a dense soil structure and texture, generally including soils that are high in clay or organic matter content, may present soil conditions with limited oxygen amounts and air flow within the soil profile (Kaya 1990; Georgis and Gaugler 1991). Land tilling can be used as a method to facilitate air flow by modifying the soil structure of otherwise dense soils. As far as nematodes as a formulated product is concerned, the presence of interstitial spaces in formulations is beneficial to nematodes, as it supports gas exchange (Grewal and Georgis 1999).

The history of entomopathogenic nematodes and nematode culturing methods

The first record of invertebrate-parasitic nematode activity was mentioned by Aldrovandus in his 'De Animalibus Insectis' in 1623. He recorded worms emerging from dead grasshopper bodies. In 1742, the French naturalist Reaumur noted nematodes in the body cavity of bumblebees which he opened to observe egg-production. He did not know, at that stage, what he had observed, but it was later discovered to be the nematode *Sphaerularia bombi* Dufour, 1837, a wide-spread parasite of queen bumblebees (Stock 2005). Gould described the effects of EPNs on ants in 1747, and Linnaeus listed eight genera of Vermes associated with both vertebrate and invertebrate hosts in 'Systema Naturae' in 1758 (Glaser 1932; Glaser and Farrell 1935).

Over 30 families of nematodes can be associated with invertebrates, with the families being split up into five major groups, including Rhabditina, Tylenchina, Myolaimina, Spirurina and Mermithina. The most primitive group, Rhabditina, gave rise to three entomopathogenic nematode families, Oxyuridae, Heterorhabditidae and Steinernematidae, of which the latter two are the most virulent families, and of which specific species are internationally produced on a commercial scale as biological pesticides (Stock 2005).

The first record of EPN mass culture for use in insect pest control was the *in vitro* production of nematodes by Glaser (1931). In 1930, Glaser and Fox found Japanese beetles infected with nematodes on a golf course in New Jersey, USA (Glaser and Fox 1930). The nematode was later described to be *Neoplectana glaseri* Steiner, 1929. Glaser succeeded in producing sufficient numbers of nematodes on agar plates to apply them later in the field against the Japanese beetle as a biological control agent (Gaugler et al. 1992). In several of the field trials, the beetle population was successfully controlled (Glaser 1931). Initially, Glaser was unaware of the important role that the symbiotic bacteria played in the killing of insects and in the *in vitro* production of EPNs. However, what could have been the start of a biological control movement was not, because of the prevailing success of pesticides during the period concerned. Renewed interest in biological control surfaced during the 1960s upon recognition of the damaging effects that pesticides might have on the environment (Griffin et al. 2001).

The first species to be used for the successful control of an insect pest 32 years ago was *S. carpocapsae*, by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia (Divya and Sankar 2009). It was commercially used to control the black vine weevil, *Otiorhynchus sulcatus* (Fabricius) in ornamentals and the currant borer moth, *Synanthedon tipuliformis* (Clerk) in blackcurrants (Bedding and Miller 1981). Today, several nematode species, such as *S. carpocapsae* Weiser, 1955; *S. feltiae* Filipjev, 1934; *S. scapterisci* Nguyen and Smart, 1990; *S. glaseri* Steiner, 1929; *S. riobrave* Cabanillas, Poinar and Raulston, 1994; and *H. bacteriophora* are produced and applied against insect pests worldwide (Ehlers 1996).

Nematodes are cultured using either *in vivo* or *in vitro* technology. *In vivo* technology involves the inoculation of a susceptible insect host with the desired nematode strain to be replicated. EPNs can readily be reared in insect hosts for the local market, to supply inoculum for small-scale field trials, and for the maintenance of laboratory cultures. As soon as higher numbers of nematodes are required, for example with orchard application, *in vitro* production is a more practical option than *in vivo*, as large numbers of IJs can be efficiently produced in fermenting tanks using the former method (Smart 1995; Ehlers 2005).

In vivo production is a low-tech, albeit labour-intensive process, and is easily implemented in research laboratories, cooperations, cottage industries and in other areas where a lack of capital outlay and technical expertise limit the adoption of *in vitro* production processes. Compared to *in vivo* production, *in vitro* production is a highly mechanised, capital-intensive, high-tech process, ideal for commercial mass-production of EPNs (Georgis et al. 2006). Nematodes have been commercially developed in North America, Europe, Australia and Asia for the control of a vast array of pests, ranging from pests occurring in greenhouses to those occurring on golf-course turfs.

Advances in production technology during the last 20 years have facilitated positive progression from solid state *in vitro* culture, developed by Bedding in 1981, to *in vitro* liquid culture technology that abides by economies of scale and which is currently used to produce nematodes on a commercial scale. *In vivo* production, in contrast, lacks economies of scale and, in order to reduce production costs, it was opted to use *in vitro* culture, even from the first application of nematodes as biocontrol agents against the Japanese beetle (Glaser 1931; Ehlers and Peters 1995). Such a technique has since been used to produce commercially available nematode products (Ehlers 2005).

In vivo culturing does, however, still play a very important role in cottage industries and in developing countries where labour is still relatively inexpensive compared to that of first-world countries. Biotechnological equipment and technical expertise regarding the subject is limited in South Africa and the commercial *in vitro* production of nematodes has yet to see the light of day (Gaugler 2002). In the interim, *in vivo* culturing methods are used in laboratories for experiments and small-scale field trials. Hopefully, *in vivo* production can be used as a stepping-stone that could soon lead to the development of *in vitro* culturing of indigenous nematode species for the commercial market in South Africa.

***In vivo* culturing of nematodes**

One of the prerequisites for a biological control agent to be successful is the capability to replicate it artificially in high numbers, and to be able to formulate it into a product with a reasonably long shelf-life of three to six months. *In vivo* nematode production is based on the White trap method, devised in 1927 by White and later reconstructed by Dutky et al. (1964), described by Poinar (1979) and modified by Lindegren et al. (1993) and Kaya and Gaugler (1993). The method involves the natural migration of IJs away from the infected host cadaver into, and the entrapment in, a surrounding water layer, from where it is harvested. High-quality nematodes are produced in this manner. In comparison, some studies have shown that *in vitro* rearing decreases the efficacy and persistence of *H. bacteriophora*

(Gaugler and Georgis 1991; Nandini et al. 2008). This, however, is a topic of debate, since alternative literature states otherwise (Glaser and Farrell 1935; Gaugler and Bilgrami 2004).

Hosts

The first step in the *in vivo* production process entails selecting a susceptible host. The most general and widely used host is the greater wax moth larva (WML). WML are a major pest in apiaries and cause severe damage to stored and unattended honeycombs. WML are highly susceptible to nematodes, are widely available, and can be easily reared on artificial diets within a relatively short time. The late-instar larvae produce sufficient numbers of nematodes to make their use feasible for *in vivo* production (Flanders et al. 1996; Hazir et al. 2003). Another promising host for nematode production is the yellow mealworm, *Tenebrio molitor* (L.) (Blinova and Ivanova 1987; Shapiro-Ilan et al. 2002). Mealworms are general decomposers and pests on poultry farms and in grain storage facilities. In most aspects they measure up to wax moth larvae as being stellar hosts, yet they are less susceptible to nematodes compared to the wax moth larvae and also produce fewer nematodes per host (Shapiro-Ilan et al. 2004).

Alternative and novel *in vivo* hosts that have been tested for cultivation of nematodes include: silkworms, *Bombyx mori* (L.) (Zaki et al. 2000; Han et al. 2003); the root grub, *Holotrichia serrata* (Fabricius); cotton bollworm, *Helicoverpa armigera* (Hübner); rice moth, *Corcyra cephalonica* (Stainton) (Ali et al. 2008); and bollworms, *Helicoverpa virescens* (Fabricius) and *Spodoptera exigua* (Hübner) (Elawad et al. 2001). Nematodes cultured in hosts within the nematodes' natural host range have proved to be of superior quality. If taken into consideration when selecting a host, the factor could further enhance nematode efficacy. It should, however, be noted that the possibility exists that nematodes could adapt to those hosts on which they are reared. In order to overcome the possible development of strain deterioration, fresh nematode genetic material can be introduced, or nematodes could be cryopreserved (Shapiro-Ilan et al. 2004; Stokwe 2009).

Diets

In vivo production requires a constant and reliable source of host insects. Imperative to the rearing of high-quality hosts and nematodes is the selection of an artificial host diet that would support development of the entire life cycle of the host, and outperform other diets in terms of host yield production, weight accumulation and developmental rate of hosts

(Cohen 2004). Comparative costs of host diets and the ability of potential hosts to proliferate on artificial diets are some of the important factors to take into consideration before selecting a host for *in vivo* nematode production. Wax moth larval diets generally include a mixture of ingredients like glycerol, yeast, milk powder and beeswax, while mealworm diets contain variations of a bran-based diet (Dadd 1966; Sarin 1972; Sarin and Saxena 1975; Edwards and Abraham 1985; Strzelewicz et al. 1985; Bhatnagar and Bareth 2004; Coskun et al. 2006; Lee et al. 2007; Birah et al. 2008; Rice and Lambkin 2009).

Inoculation of insect hosts with nematodes

The next step in the *in vivo* production process is the inoculation of insect hosts. Different methods of inoculation have been tested with varying success in obtaining maximum latent infections. These include immersion of hosts into a nematode suspension, the spraying or pipetting of nematodes onto hosts, or adding nematodes directly to the host diet (Shapiro-Ilan et al. 2002).

The efficacy of *in vivo* nematode production relies heavily on consistently high infection rates. Therefore, any parameter that may influence infectivity by IJs should be optimised. These parameters include: nematode concentration applied; inoculation method used; host density; humidity; and temperature (Woodring and Kaya 1988; Grewal et al. 1994b; Shapiro-Ilan et al. 2002). Time required to perform inoculation is also a deciding factor when selecting an inoculation method, as it may influence labour costs and production output (Shapiro-Ilan et al. 2002).

Measures to enhance nematode infectivity indirectly through host and nematode manipulation can be applied in addition to optimising inoculation methods. For example, imposing physical stress on an insect host can compromise the host's defences by making it more susceptible to nematode infection. Exposure of mealworms to temperature extremes and dehydration stress has proven to increase the infection of insect larvae by *H. bacteriophora* (Brown et al. 2006). It is believed that heat shock inhibits the ability of the insect to control the muscles that are responsible for the closing of bodily orifices, which, consequently, leads to higher nematode penetration rates. In mealworms, heat shock may also be responsible for the denaturation of an anti-microbial protein that is responsible for having a negative impact on the functioning of bacterial symbiont *Photorhabdus* spp. Thomas and Poinar, 1979 (Brown et al. 2006). Pre-inoculation chemical treatment of nematodes using certain elements like manganese, magnesium and manganese sulphate has proven to increase virulence in *S. carpocapsae* and *H. bacteriophora* (Jaworska et al. 1999). A combination of the above-mentioned techniques can, in addition to assisting in achieving consistently high infection

rates, also increase the potential of certain insects to be suitable hosts, which would not have been the case without the addition of physical and/or chemical stress.

Formulation of nematodes

Knowledge of nematode physiological chemistry, ecology, behaviour and the liquid culturing of nematodes has made the mass production of nematodes a feasible process to implement (Georgis and Kaya 1998). Even though nematodes can be produced in high numbers, it is vital to keep them stable during storage and application to ensure high efficacy in the field. Formulating nematodes facilitates extended product storage and transportation, which are two main objectives for commercialisation.

According to Jones and Burges (1998), formulation can be defined as an aid in preserving organisms, which helps to deliver them to their targets and, once there, assists in improving their mode of action. Generally, formulations are a combination of an active ingredient and a non-active ingredient. In this case, the nematode would be the active ingredient and an inert material, like clay, would be the non-active ingredient (Gaugler 2002). One major difference in the formulation of nematodes compared to chemical insecticides is that biological activity of an organism with high oxygen, moisture and temperature requirements has to be maintained until the product is applied in the field (Jones and Burges 1998). The ideal formulation would adhere to the following four characteristics: stabilisation of nematodes during production, distribution and storage; ease in handling and application of nematodes; protection of nematodes from harmful environmental factors; and enhancing nematode activity at the target site. In practice, a perfect formulation does not exist, but measures to improve current formulations, with the aim of achieving perfection in a cost-effective manner, contributes significantly to the development of superior products.

The first attempts made at formulating EPNs in 1979 resulted in a formulation with a maximum shelf-life of 30 days (Georgis and Kaya 1998). Solid and liquid carriers like sponge, vermiculite and peat were some of the formulation carriers used, but they required refrigeration and had to remain moist. With advances being made in coming to a better understanding of the temperature, oxygen and moisture requirements of different nematode species, formulation types were adapted and modified to support the pH, temperature and osmolarity requirements of specific nematode genera and/or isolates (Strauch et al. 2000). Such advances gradually led to the transition from wet formulations to partially desiccated formulations, like absorbent clays and water-dispersable granules. The development of drier formulations was triggered by the discovery that a slow rate of water loss conserves lipid reserves, which, in turn, influences IJ

viability, pathogenicity and ability to tolerate higher temperatures over time. Gradual water loss in the IJ leads to partial anhydrobiosis, with the nematodes coming to be referred to as quiescent anhydrobiotes (Womersley 1990a, b).

Some formulations considered to facilitate partial anhydrobiosis are polyacrylamide gel (Bedding and Butler 1994); powders (Bedding 1988); granules (Capinera and Hibbard 1987; Connick et al. 1993); and water-dispersible granules (Georgis et al. 1995; Silver et al. 1995; Grewal and Georgis 1999; Grewal 2000). The addition of pesticide formulation ingredients and such additives as adsorbents, preservatives and binders can further increase shelf-life (Georgis and Kaya 1998). Other formulations, such as calcium alginate plastic screens, play a prominent role in restricting nematode movement. This type of formulation could be beneficial in preserving the lipids of nematodes characterised by a cruising foraging behaviour, and, in effect, extend shelf-life.

In order to be commercialised, chemical pesticides need to meet a two-year shelf-life requirement. Although steady progress has been made in the formulation design of biological control agents, the development of a formulation that is able to sustain nematode activity for a period of even one year has not yet been achieved (Gaugler 2002). Further research to better understand nematode physiology and behaviour is necessary in order to improve formulations that contain nematodes. The development of more technologically advanced nematode formulations in such third-world countries as South Africa is still in the initial developmental phases due to lack of research and expert knowledge, and to the secrecy of information on the topic.

Cost

The production and storage costs associated with EPNs tend to be higher than those that are associated with chemicals. It was stated in 1991 that insect pest control using EPNs as biological agents could cost 10-60% more than chemical insecticides (Smart 1995). Since then, tremendous advances have been made in the production of EPNs, which have halved the costs of *in vitro* mass production (Ehlers 2001). Even though the short-term initial costs are high, in some instances, recycling of nematodes in nearby susceptible hosts after application takes place, with the result that nematodes control the insect pest for a prolonged period post-application. This could lead to lower costs in the long term (Smart 1995). According to Grewal and Georgis (1999), the price of some nematode products is comparable to that of standard insecticides in certain markets. However, the positive impact of nematodes on the control of pest insects without harming either the environment or humans cannot be measured in monetary terms only.

The production costs of EPNs need to be further reduced to ensure branching out from the high-value crop market into the low-value crop market. Improvement of the production process, genetic improvement and attaining a better understanding of nematode reproduction biology, as well as favourable regulation requirements, will assist in achieving the stated goal (Ehlers 2001).

Quality control

Since the appearance of nematode biopesticides on the market, there have been persistent concerns about the quality of such products. Although the efficiency of a production process is important, of equal importance is maintaining nematode quality (Georgis and Hague 1991). Lack of viability and insufficient activity of nematodes after application can easily destroy market perception and the acceptance of nematode-based products.

To ensure consistent performance of formulated nematode products in the field, quality control measures are implemented in the production process. Standardised quality tests should be conducted during production and storage, after delivery, and both before and after application, in order to certify that nematodes are in an optimal condition. Quality control measures include: determination of the percentage of dead nematodes in a batch; establishment of the movement ability of IJs; heat shock assays; insect bioassays; and neutral lipids estimation (Grunder et al. 2005). However, field efficacy is considered to be the ultimate measure of EPN quality (Gaugler et al. 2000).

Conclusion

The *in vivo* production of EPNs for the control of insect pests has been studied extensively. However, enhancing and streamlining existing practices can contribute towards a more effective, cost-efficient and practical production process. Selecting hosts that are highly susceptible to the specific nematode strain, and optimising their diets, can contribute to achieving higher infectivity rates and a higher nematode yield. Optimising the inoculation step in the production process can further enhance infectivity and reduce the amount of labour-intensive removal of uninfected hosts required. Selection of the most practical and effective formulation for the storage of nematodes will ensure the delivery of virulent nematodes to the area of application. Throughout the production process, such abiotic parameters as temperature, humidity and oxygen availability, which are critical to the survival of nematodes, can be adjusted to create an optimal environment for nematode activity and survival.

With the right quality control measures in place, high-quality endemic nematodes can be produced on a small scale for research laboratories, niche markets and organic grower cooperatives. The current situation in agriculture, favouring the adoption of environmentally benign approaches, is highly conducive to the use, and inherently to the effective production, of EPNs.

Aims of the current study

1. Cost-effective culturing of *Tenebrio molitor* L. (yellow mealworm) and *Galleria mellonella* L. (greater wax moth) larvae, through selection of respective superior diets.
2. Improving of nematode infectivity by selection of an effective inoculation method and manipulation methods for both nematode and insect host.
3. Comparing of various customised nematode formulations and selecting of the most promising formulation according to results obtained, using a specific nematode quality protocol.

Chapters 3, 4 and 5 are in the style of publishable manuscripts and, for that reason, a certain amount of repetition was unavoidable.

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Chapter 2

Rearing of wax moth and mealworm larvae for *in vivo* nematode production

Introduction

The establishment and maintenance of insect colonies is vital to the advancement of biological insect-control techniques and has lately been used especially for sterile insect release (SIR) purposes (Singh 1983; Bartlett 1984). A large number of hosts are required for the *in vivo* mass production of entomopathogenic nematodes (EPNs), since the insect host serves as a bioreactor, in which the nematodes multiply (Sharma et al. 2011). Wax moth larvae (*Galleria mellonella* L.) and mealworms (*Tenebrio molitor* L.) were selected as nematode hosts. The aim of the culture method concerned is to ensure a continuous supply of sufficient numbers of wax moth larvae (WML) and mealworms (MW) of high quality.

Material and methods

Rearing of wax moth larvae

Wax moth eggs and larvae obtained from the initial laboratory colony were placed on top of an artificial diet in well-ventilated plastic containers measuring $11 \times 11 \times 7.5$ cm (length \times width \times height). The artificial diet consisted of 118 g wheat flour, 206 g wheat bran, 118 g milk powder, 88 g brewer's yeast, 24 g wax powder, 175 ml honey and 175 ml glycerol. The plastic containers were modified by inserting mesh screen in the lid to facilitate air and heat exchange with the aim of avoiding condensation. The life cycle of WML includes an egg, larva, pupa and moth stage and development took place at an average temperature of 26°C and at an average relative humidity (RH) of 55% (Warren and Huddleston 1962; Mikołajczyk and Cymborowski 1993; Cymborowski 2000). Moths were regularly removed from the diet as they hatched, one to two weeks after pupation. They were then placed together in glass jars containing pleated wax paper, which served as an ovi-position site for female moths. The moths started laying eggs three to five days after emergence and had a life span of one to two weeks at 26°C. Eggs were regularly removed from the paper with a razor blade and transferred to a plastic container, containing 1 kg of fresh artificial diet. Eggs hatched within four to five days after being laid. The diet was replenished as needed to ensure sufficient nutrient availability for larvae. The larval stage (Fig.

1A) lasted between 20-40 days, after which larvae not used in experiments pupated, and the life cycle, which took six to seven weeks, was repeated.

Rearing of mealworms

MW larvae (Fig. 1B), which were obtained from a laboratory culture, were reared on wheat bran as culture medium in various sized wooden culture boxes covered with a solid lid to limit light penetration (Cotton 1927; Arendse and Vrinis 1975). Bran was frozen before use to prevent mite infection. Sufficient wheat bran (1 kg) was added to facilitate burrowing for larvae and adult beetles. Carrots, wiped with 96% ethanol prior to use, were placed on top of the diet to serve as water source, and were replenished as required during the life cycle, which varied between 12-16 weeks. Wooden culture boxes containing the egg, larva, pupa and beetle stages of MW were placed in a temperature-regulated room at an average temperature of 26°C and at an RH of 55%. Depending on the size of the colony, bran was replenished once every two weeks and carrots were added every week. At least once every three months, or as soon as the diet seemed to become moist, beetles and larvae were separated from eggs and frass, using a sieve of 1200 µm. Fresh diet and carrots were then added to the culture box, containing beetles and larvae. Frass containing the eggs was placed in a separate container, to which a fresh diet and carrots were also added, available for consumption by the next generation of MW.

During periods that MW and WML were not needed in large numbers for experiments, the culture boxes were moved to a 14°C culture room to slow down the developmental rate of the hosts.



Fig. 1 (A) Wax moth larvae and (B) mealworm larvae used as insect hosts for the *in vivo* production of *Heterorhabditis zealandica* and *H. bacteriophora*.

Conclusion

The culture method applied ensured continuous availability of WML and MW in various life stages. Basic equipment was used and the diets selected were cost-efficient. The culture method used was easily reproducible, practical and could produce nematodes on a small scale over an indefinite period of time. Although slight contamination problems were experienced in initial MW diets, due to the addition of carrot, the problem could be overcome by removing the carrot before rotting, or by replacing the carrot with an alternative water source, for instance paper towels wetted with tap water. However, prevention of contamination through the implementation of effective sanitary measures is key to a successful rearing programme (Sikorowski 1984).

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Chapter 3

Cost-effective culturing of *Galleria mellonella* (greater wax moth larvae) and *Tenebrio molitor* (yellow mealworm larvae), and nematode production in various hosts

Abstract

Wax moth larvae (*Galleria mellonella* L.) and yellow mealworm larvae (*Tenebrio molitor* L.) were reared on five different diets respectively, to determine which diet resulted in the highest increase in insect larval weight. Wax moth eggs were placed on each diet and larvae that developed therefrom were weighed after 51 days. A diet containing 118 g wheat flour, 206 g wheat bran, 118 g milk powder, 88 g brewer's yeast, 24 g wax powder, 175 ml honey and 175 ml glycerol, produced the heaviest larvae, with an average weight of 0.19 g per larva. Mealworms were weighed 62 days after adult beetles were placed on the various diets. The heaviest larvae were produced on a diet consisting of wheat bran only, with each larva weighing, on average, 0.0154 g. Nematode production of *Heterorhabditis zealandica* and *H. bacteriophora* using wax moth, mealworm, codling moth (*Cydia pomonella* L.) and false codling moth (*Thaumatotibia leucotreta* M.) larvae were determined, as well as was the correlation between the weight of the host larvae and that of the nematode progeny produced. Wax moth larvae produced the highest number of *H. zealandica* and *H. bacteriophora* per g host. The mean number of *H. zealandica* and *H. bacteriophora* infective juveniles produced were 1 459 205 and 1 898 512 per g host. The mean number of *H. zealandica* produced per g codling moth and false codling moth was 57 582 and 192 867, respectively. Lower mean numbers were produced for codling moth and false moth when using *H. bacteriophora*, with 39 653 and 97 652 nematodes produced per g of the respective hosts. A significant positive linear relationship existed between weight of wax moth and that of mealworm larvae and the number of nematodes produced. Using frozen hosts of wax moth and mealworm larvae, comparable reproduction of nematodes was only found with mealworm larvae.

Introduction

Entomopathogenic nematodes (EPNs) are facultative parasites of insects and have been successfully used for the biological control of various pest insects, especially in the European and American market (Ehlers 1996). They occur in

soils worldwide and specific species are mass cultured and formulated for use against a wide range of insect pests (Ehlers 2001; Ehlers and Shapiro-Ilan 2005). They can be mass-produced using either *in vivo* or *in vitro* techniques. For small-scale application *in vivo*, culturing is an economically feasible method of nematode production (Gokte-Narkhedkar et al. 2008). *In vivo* nematode production involves culturing of nematodes in a susceptible insect host. The availability of a constant and reliable source of host insects throughout the year can only be achieved by laboratory culturing.

Selecting a suitable host for *in vivo* nematode production is key to the success of the production process and greatly affects nematode yield and costs (Ehlers and Shapiro-Ilan 2005). Host susceptibility, host availability, ease and cost of rearing the host, and quality of nematodes produced by the host are all parameters to take into consideration when selecting a suitable host (Blinova and Ivanova 1987, Hatab et al. 1998; Shapiro-Ilan and Gaugler 2002; Shapiro-Ilan et al. 2004). The developmental stages of hosts also play an important role in their susceptibility to nematodes (Simoes and Rosa 1996; Hatab et al. 1998; Ehlers and Shapiro-Ilan 2005). Insects with specific physical characteristics, such as oral filters, narrow mouthparts, constricting muscles or sieve plates covering spiracles, like wireworms, grape phylloxera, fire ants or corn rootworms, make nematode infection impossible and are not ideal hosts for nematode culturing (Gaugler and Bilgrami 2004).

Optimal rearing conditions are needed for the culturing of sufficient numbers of high-quality insect hosts. Abiotic factors like temperature, humidity, light and aeration requirements influence the growth, development and behaviour of hosts (Owens 1984). In an enclosed rearing facility, environmental conditions can be manipulated to increase the efficacy of host production. Temperature can have an impact on every developmental stage in the life cycle of an insect (Cymborowski 2000). By increasing the ambient temperature, an increase in the growth rate of an insect generally occurs, accordingly shortening the life cycle period, and increasing cost efficiency (Hagstrum and Milliken 1988; Shapiro-Ilan et al. 2004). Furthermore, temperature can increase or decrease fecundity and larval weight (Warren and Huddleston 1962; Hagstrum and Milliken 1988; Jyothi and Reddy 1996). Lower temperatures generally lead to a reduced developmental rate (Mikołajczyk and Cymborowski 1993). Humidity has been shown to have significant effects on the hatchability, pupation and mortality of insect hosts (Hagstrum and Milliken 1988; Hussain et al. 2011). Insects that are nocturnal, like wax moth larvae (WML) moths, are highly active during the night, thus rearing them in the dark could increase mating and reproduction (Warren and Huddleston 1962). Air movement is also a critical factor to consider in rearing facilities. Too much air movement can lead to drying out of insect diets, while a lack of air movement can lead to humid and unfavourably low oxygen conditions, which are ideal for unwanted microbial growth (Griffin 1984).

Formulation of an artificial diet is fundamental to the process of laboratory insect rearing. The *in vivo* production process is labour intensive and does not adhere to economies of scale (Friedman 1990; Shapiro-Ilan et al. 2004; Divya and Sankar 2009). Therefore, with diet being arguably the most costly factor in the *in vivo* culturing process (Divya and Sankar 2009), it is of extreme importance that diets be optimised to the extent where high-quality insect hosts, in sufficient numbers, are produced using an economically viable diet. Bogdanov developed the first artificial diet in 1908 to rear blowflies, *Calliphora vomitoria* (L.) (Singh 1977). Since then, great advances have been made in diet formulation, especially for Lepidoptera, Coleoptera and Diptera (Singh 1984), by adding essential and non-essential nutrients, conforming to the nutritional requirements of hosts, and thus expanding the pool of insects that could be reared on artificial medium. However, only a few diets have been entirely successful in replacing the natural diet of host insects (Cohen 2004).

Artificial diets can be divided into two categories: the holidic diet and the meridic diet. According to Cohen, the holidic diet consists of a diet in which very few of the ingredients are chemically pure or defined, whilst all of the ingredients included in a meridic diet are thus controlled (Cohen 2004). When developing an artificial diet, chemical composition, diet nutrition and feeding behaviour of the insect should be taken into consideration, because such factors can determine whether a diet will succeed or not (Singh 1977).

In a study by Elawad et al. (2001) and Ali et al. (2008), where nematode yield for various lepidopterous hosts were compared, great differences occurred in the final EPN numbers obtained per insect host. Calculating the number of nematodes produced per host would assist in determining, in advance, how many larvae need to be reared for a specific field trial, experiment or formulation. Knowing the nematode production potential of a particular insect host would further assist in selecting a suitable host, because, ultimately, host selection rests on nematodes produced per cost of susceptible insect (Ehlers and Shapiro-Ilan 2005).

The first objective of this study was to compare various cost-efficient host diets, with the aim of selecting one superior diet that would support development of the host's entire life cycle, produce high-quality hosts and outperform the other diets in terms of average weight accumulation of the host reared on the specific diet. The second objective was to determine the number of infective juveniles (IJs) produced by two local nematode species, using various insect hosts, including laboratory-reared and commercially-produced insects.

Materials and methods

Source of host insects

The starter colony of WML used for nematode production was obtained from an existing laboratory culture that was kept at room temperature in dark, aerated plastic containers. The initial mealworm larvae (MW) were obtained from a laboratory culture reared in wooden boxes that were also kept at room temperature (Chapter 2).

Late instar codling moth (CM) larvae were obtained from Entomon, a sterile insect release codling-moth-rearing facility in Stellenbosch, South Africa. False codling moth larvae (FCM) were obtained from River Bioscience in Summerstrand, Port Elizabeth, South Africa, and received via post in ventilated glass bottles containing diet and late instar larvae. Larvae were immediately used upon reception from the respective facilities.

Source of nematodes

The two endemic nematode isolates used in this study were *Heterorhabditis bacteriophora* Poinar, 1976 isolate SF351 and *Heterorhabditis zealandica* Poinar, 1990 isolate SF41 (Table 1). These two strains were obtained from the EPN collection at Stellenbosch University and were found during local surveys conducted in South Africa (Malan et al. 2006) (Table 1).

Table 1 *Heterorhabditis* species, isolate numbers, GenBank accession numbers, locality where it had originally been sampled and preferred habitat.

Species	Isolate number	GenBank accession number	Locality	Habitat
<i>H. bacteriophora</i>	SF351	FJ455843	Wellington	Vineyard
<i>H. zealandica</i>	SF41	EU699436	Patensie	Natural vegetation

(Malan et al. 2006)

Culturing and storage of nematodes

Nematodes used as inoculum for the experiments were cultured at 26°C using WML according to White's (1927) method, which was later amended by Kaya and Stock (1997). Ten infected WML were placed on 90-mm Petri dishes

lined with filter paper (Whatman No. 1) and inoculated with 200 nematodes / 50 µl distilled water per host. Nematodes were harvested according to the method used by Kaya and Stock (1997). The IJs harvested from White (1927) traps were stored horizontally in vented 160-ml culture flasks in 50 ml of distilled water at 14°C in the dark. Flasks were shaken on a weekly basis to facilitate proper aeration. Inoculum was used within a month after harvest and checked for viability before use in experiments (Gaugler et al. 2000).

Diet formulation for the production of wax moth larvae and mealworms

Dry ingredients, which were included in diets for both WML and MW, were sterilised by microwaving them for one minute before use, and stored at -20°C (Rajagopal 2009). Diets for both WML and MW were prepared by mixing ingredients together at room temperature (25°C). Each diet was evenly distributed into 20 square plastic containers measuring 11 × 11 × 7.5 cm and sealed with lids fitted with steel mesh screens. Containers were wiped before use with 75% ethanol. A total number of 20 containers were used for each of the five diets ($n = 100$). Each container received 50 g of the specific diet combination.

Diets used for the production of wax moth

The five diet recipes (Table 2) used for WML were adaptations from diets by Poinar (1975); Strzelewicz et al. (1985); Bhatnagar and Bareth (2004); Coskun et al. (2006); Lee et al. (2007); Meyling (2007); and Birah et al. (2008). Diet 1, which originally contained corn as an ingredient, was modified by substituting corn with wheat bran, to avoid the possible negative effects corn containing the Bt-gene might have on larval development (Stenekamp 2011).

Table 2 Ingredient composition and costs per kg of various wax moth larval diets.

Diet	Ingredients	Amount per kg	Cost per kg (ZAR)*
Diet 1	Wheat flour	118 g	
	Wheat bran	206 g	
	Milk powder	118 g	
	Brewer's yeast	88 g	
	Wax powder	24 g	
	Honey	175 ml	
	Glycerol	175 ml	39.23
Diet 2	Wheat bran	570 g	
	Yeast	190 g	
	Honey	190 ml	27.95
Diet 3	Wheat bran	570 g	
	Yeast	190 g	
	Glycerol	190 ml	27.42
Diet 4	Cerelac TM baby cereal	240 g	
	Wheat bran	240 g	
	Brown bread flour	240 g	
	Wheat germ	95 g	
	Brewer's yeast	95 g	
	Glycerol	36 ml	
	Honey	36 ml	36.03
Diet 5	Pedigree TM Junior dry dogfood	345 g	
	Rolled oats	85 g	
	Wheat bran	85 g	
	Brewer's yeast	35 g	
	Honey	190.5 ml	
	Glycerol	165 ml	34.02

*Costs based on 2010 retail prices.

Diets used for the production of mealworm

The five MW diets (Table 3) were adapted from Barke and Davis (1967); Elowni and Elbiha (1979); Rueda and Axtell (1996); Rice and Lambkin (2009) and Shapiro-Ilan et al. (2008).

Table 3 Ingredient composition and costs per kg of various mealworm diets.

Diet	Ingredients	Amount per kg	Cost per kg (ZAR)*
Diet 1	Wheat bran	760 g	12.92
	Layer pellets	170 g	
	Yeast	70 g	
Diet 2	Wheat bran	950 g	12.57
	Yeast	50 g	
Diet 3	Maizena (corn flour)	333 g	32.61
	Yeast	333 g	
	Wheat bran	333 g	
Diet 4	Yeast	200 g	46.27
	Sunflower oil	80 ml	
	Potato starch	700 g	
Diet 5	Wheat bran	1000 g	9.97

* Costs based on 2010 retail prices.

Evaluation of the different diets on wax moth production

For the five WML diets tested, ten freshly harvested WML eggs were placed on top of each diet inside a wax paper cup. The containers were then placed in a temperature-regulated room with an average temperature of 26°C, at 55% RH in the dark. After 51 days, larvae were removed from the diet, counted and weighed individually. The experiment was repeated on a different test date.

Evaluation of the different diets on mealworm production

For the five MW diets, 10 adult beetles, younger than three days old, were placed on top of the 50 g of diet, and 50 g of carrots were added as a water source. The containers were placed in a temperature-regulated room with an average temperature of 26°C, at 55% RH in the dark. After initiation of the experiment, carrots were added twice, once on day 21 and later on day 29. Larvae were removed from the diet after 62 days. A sieve with a mesh size of 1200 µm was used to separate larvae from the diet. Larvae produced within each container of the same diet were weighed together. The average number and weight of MW larvae was then determined for each diet.

Determining the number of nematodes produced in four insect hosts

The last larval instar of WML, MW, CM and FCM, were used as hosts to determine the number of *H. zealandica* and *H. bacteriophora* IJs produced from each host. In the case of WML and MW, each host was weighed individually and one larva was placed on a filter-paper-lined 90-mm Petri dish and inoculated with 50 µl distilled water containing 200 nematodes. For CM and FCM, larvae were weighed in groups of ten, placed on filter-paper-lined Petri dishes and inoculated with 2 000 nematodes. Thirty Petri dishes per host were used for each of the two nematode species. The Petri dishes containing the inoculated hosts were then placed in sealed plastic containers (30 × 20 × 7 cm), lined with moist paper towels, and incubated at 25°C for seven days for WML and MW, and for two days for CM and FCM. Larvae were then transferred to White traps containing clean, moist filter paper. White traps were placed at 26°C and at an average RH of 55% for 32 days for WML and MW, and for 14 days for CM and FCM. Nematodes were harvested from the respective hosts by decanting them into 160-ml culture flasks, and counted according to Glazer and Lewis's (2000) method. The number of IJs produced for each insect host was determined for both nematode species. The experiment was repeated on a different test date.

The number of *H. bacteriophora* IJs produced from frozen WML and MW was also determined. Individuals of the two insect hosts were placed at -20°C for three days, removed from the freezer and inoculated before onset of thawing with a concentration of 200 IJs / insect. The experimental procedure that was followed was similar to that applied to determine nematode yield for fresh WML and MW described above.

Data analysis

Statistical analysis for both the diet and nematode yield comparisons was performed making use of the Statistica Version 10 (StatSoft Inc. 2011) data analysis software system. A one-way ANOVA analysis was performed on data obtained from the different diets. Bootstrap analysis was performed on the data obtained when residuals were found not to be normally distributed. Bonferroni's method was used to determine differences between diets by means of obtaining post-hoc comparisons of means.

Data for nematode production were analysed using a one-way ANOVA. Residuals obtained, which were not normally distributed, were analysed using bootstrap analysis. In determining differences obtained among hosts regarding total nematode production, Bonferroni's method was used. Linear regression scatterplots were used to determine the correlation between weights of hosts and the number of nematodes produced.

Results

Mean wax moth larval weight per diet

Significant differences in terms of the weight of insect hosts were detected among the five diets tested ($F_{(4, 181)} = 29.663$, $p < 0.001$). Diet 1 produced the heaviest WML, with a weight of (mean \pm SE) $0.19\text{g} \pm 0.02$ per larva, whilst Diet 3 produced the lightest larvae, with a weight of $0.08\text{g} \pm 0.01$ per larva. Diet 2 produced no larvae. Significant differences in host weight were detected between Diet 1 and Diet 3 ($p < 0.001$) and between Diet 3 and Diet 5 ($p = 0.005$). WML in Diet 5 had a weight of $0.14\text{g} \pm 0.01$ per larva. No significant differences occurred between Diet 3 and Diet 4 or among Diets 1, 4 and 5 (Fig. 1).

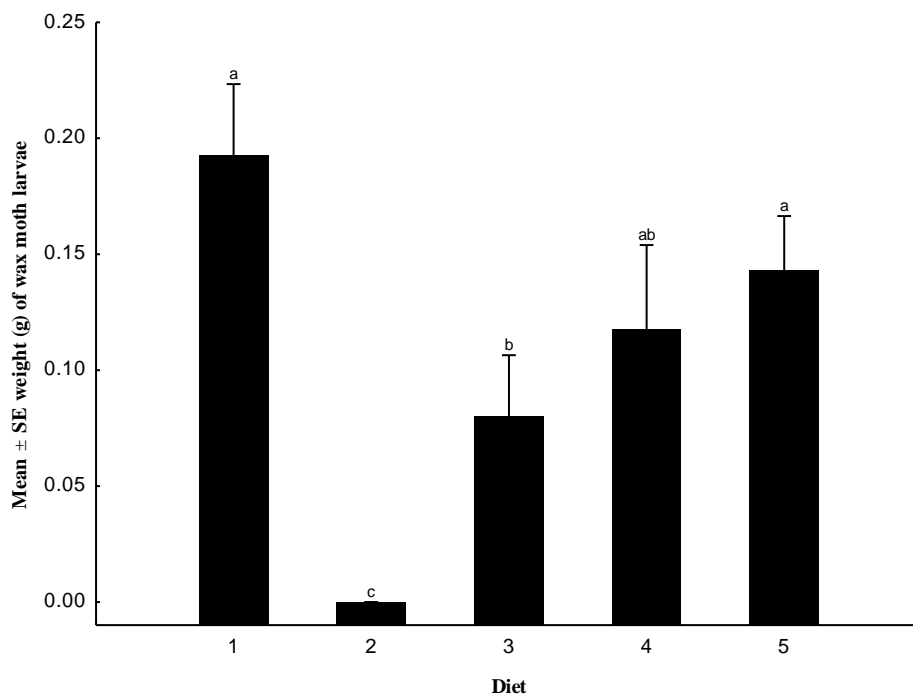


Fig. 1 The mean \pm SE (standard error of the mean) weight of individual wax moth larvae (95% confidence interval) for each of the five diets, recorded 51 days after placing eggs on the diets (one-way ANOVA; $F_{(4, 181)} = 29.663$, $p < 0.001$). Significant differences are indicated by different letters above vertical bars.

Mean mealworm larval weight per diet

Significant differences occurred with regard to the weight of MW larvae among diets ($F_{(4, 87)} = 23.114$, $p < 0.001$). Diets 1 and 2 differed significantly from each other ($p < 0.05$) as well as from Diets 3, 4 and 5 ($p < 0.001$). The average weight per larva for Diet 1 and 2 was (mean \pm SE) $0.0076\text{g} \pm 0.0008$ and $0.0048\text{g} \pm 0.0010$, respectively. Diet 2 produced larvae with the lowest mass, as opposed to Diet 5, which produced the heaviest larvae, weighing $0.0154\text{g} \pm 0.0008$ (Fig. 2).

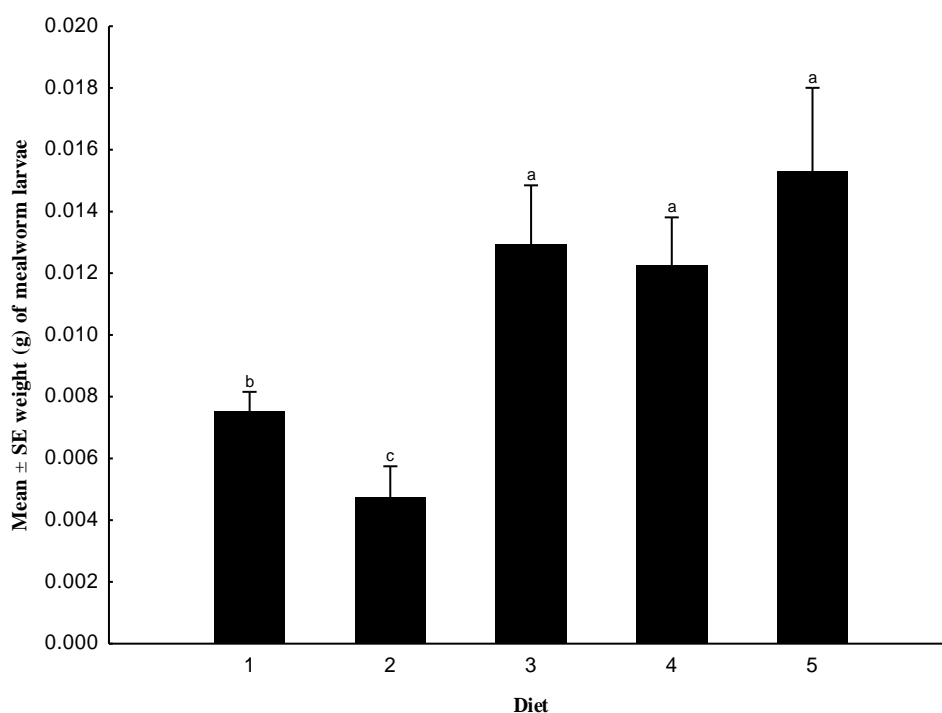


Fig. 2 The mean \pm SE weight of individual mealworm larvae (95% confidence interval) for each of the five diets, recorded 62 days after placing adult beetles on the diets (one-way ANOVA; $F_{(4, 87)} = 23.114$, $p < 0.001$). Significant differences are indicated by different letters above vertical bars.

Number of nematodes produced per g insect host

Nematode progeny production of *H. bacteriophora* in WML was the highest, compared to the other hosts (Fig. 3). The mean number of nematodes produced was $1\,898\,512 \pm 94\,355$, and differed significantly from production in the other hosts ($p < 0.001$). CM produced the lowest number of *H. bacteriophora* IJs, yielding $39\,653 \pm 8\,276$, followed by frozen WML ($68\,703 \pm 23\,301$) and FCM ($97\,652 \pm 23\,404$). Significant differences occurred between MW larvae, which produced $414\,566 \pm 67\,017$ nematodes, and all of the other hosts. MW and frozen MW showed no significant differences in nematode production ($p = 1.00$). No significant differences were, however, found in progeny production among frozen WML, CM ($p = 1.00$) and FCM ($p = 1.00$) (Fig. 3).

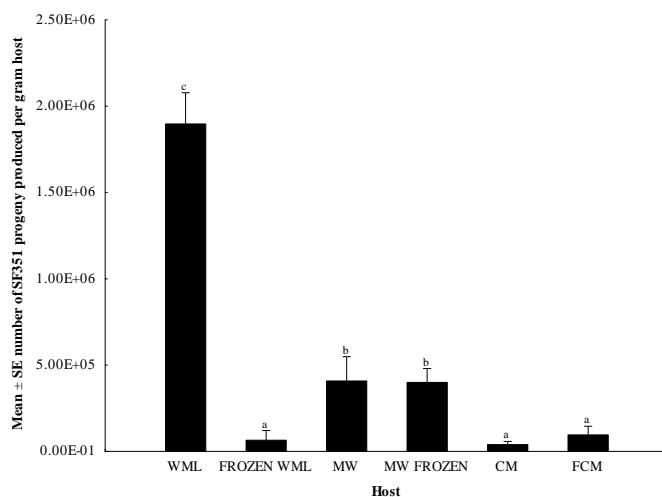


Fig. 3 The mean \pm SE number of *H. bacteriophora* (SF351) progeny produced in four different insect hosts (95% confidence interval). Hosts were inoculated with a concentration of 200 IJs / insect (one-way ANOVA; $F_{(5, 264)} = 119.51$, $p < 0.001$). Significant differences are indicated by different letters above vertical bars.

Significant differences ($F_{(3, 152)} = 33.053$, $p < 0.001$) were also detected among the number of *H. zealandica* (SF41) progeny produced per host g for all the different insect hosts (Fig. 4). The highest number of nematodes was produced in WML ($1\,459\,205 \pm 113\,670$), followed by MW ($836\,690 \pm 121\,252$), FCM ($192\,867 \pm 13\,488$) and CM, with the lowest mean number of nematodes ($57\,582 \pm 10\,026$) (Fig. 4).

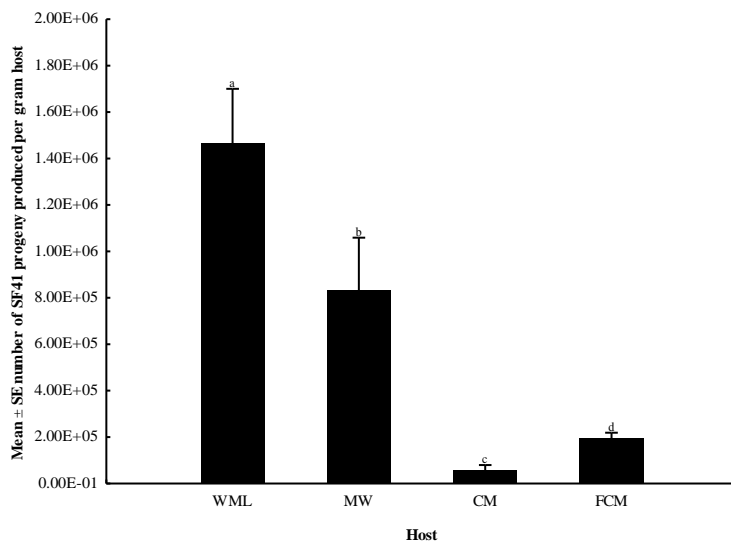
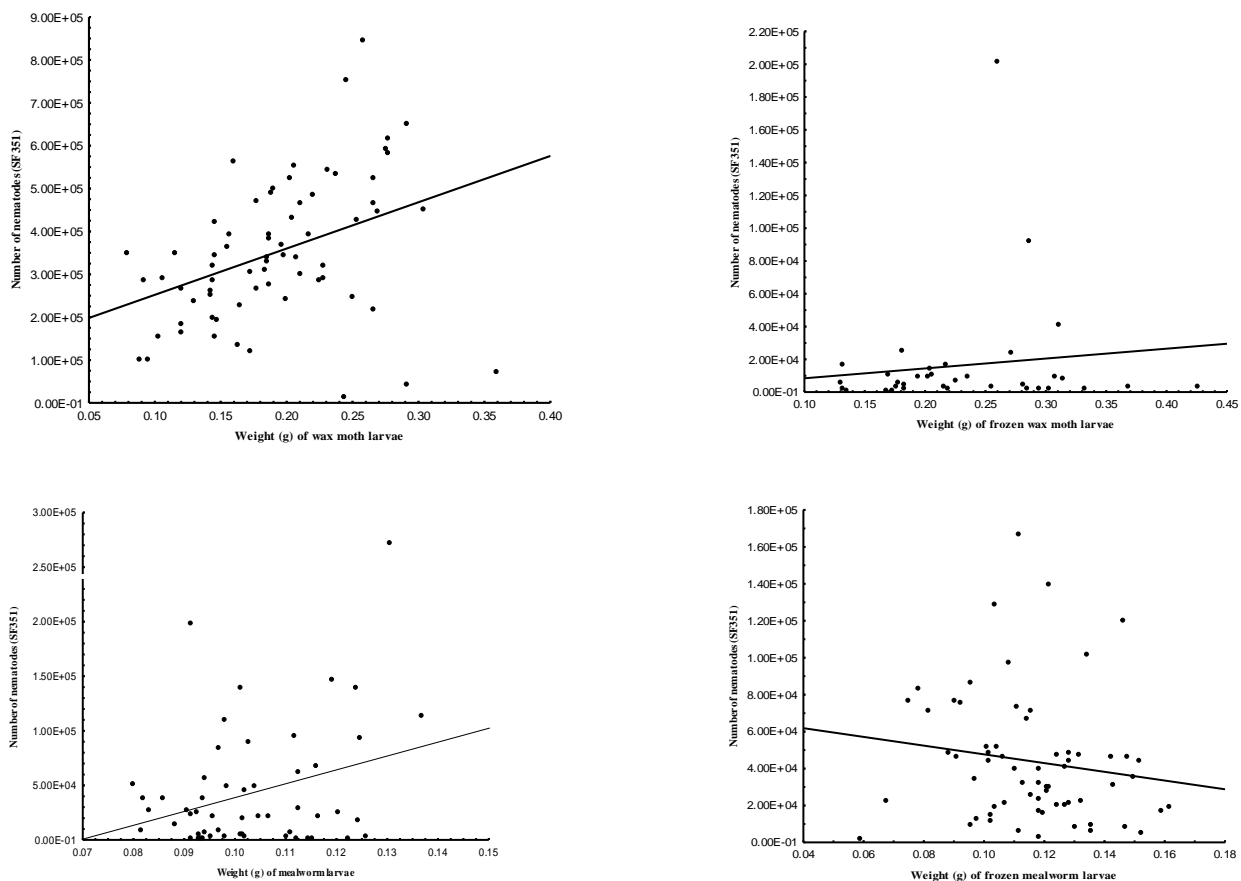


Fig. 4 The mean \pm SE number of *H. zealandica* (SF41) progeny produced in four different insect hosts (95% confidence interval). Insects were inoculated with 200 IJs / insect (one-way ANOVA; $F_{(3, 152)} = 33.053$, $p < 0.001$). Significant differences are indicated by different letters above vertical bars.

Correlation between host weight and number of IJs produced

A significant, positive, linear relationship between the weight of WML and number of *H. bacteriophora* progeny produced was found to exist ($\rho < 0.05$; $y = 1\,079\,800x + 144\,220$). The same was observed for MW ($\rho < 0.05$; $y = 1\,270\,600x - 87\,065$). However, in the case of frozen MW, an increase in the weight of frozen larvae led to a decrease in the number of nematodes produced, indicated by the negative linear relationship, $y = -236\,320 + 71\,344$. There were no significantly positive or negative linear relationships between the weights of frozen WML, CM, FCM and the number of *H. bacteriophora* progeny produced (Fig. 5).



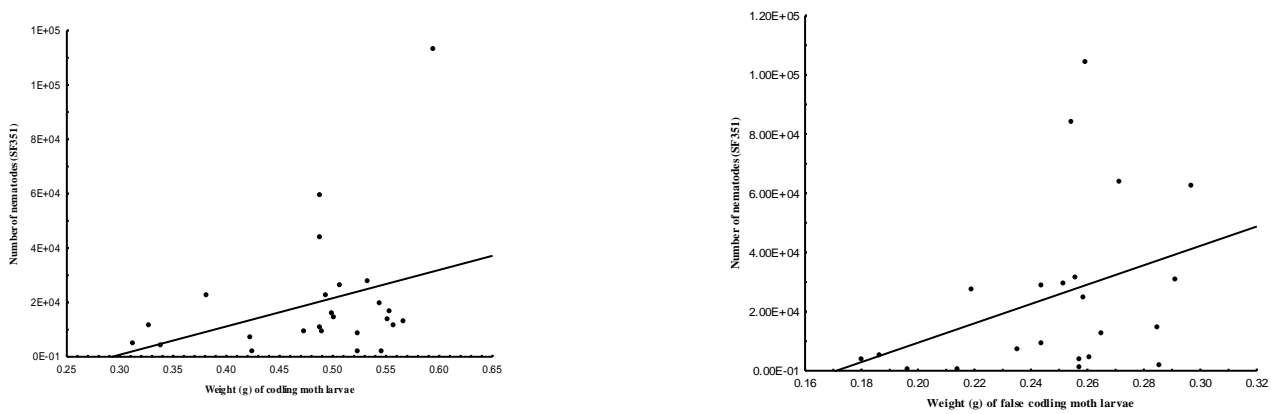


Fig. 5 Scatterplots depicting the correlation between weight (g) of hosts and the number of *H. bacteriophora* (SF351) progeny produced by each host.

The number of *H. zealandica* progeny produced in WML and MW larvae also linearly increased as the weight of the insects increased. A significant, positive correlation between the weight of WML ($p < 0.001$; $y = 2\,226\,100x - 128\,250$) and MW larvae ($p < 0.001$; $y = 1\,946\,700x - 130\,000$) was observed. The weight of CM and FCM larvae did not have a significant correlation to the number of nematodes produced (Fig. 6).

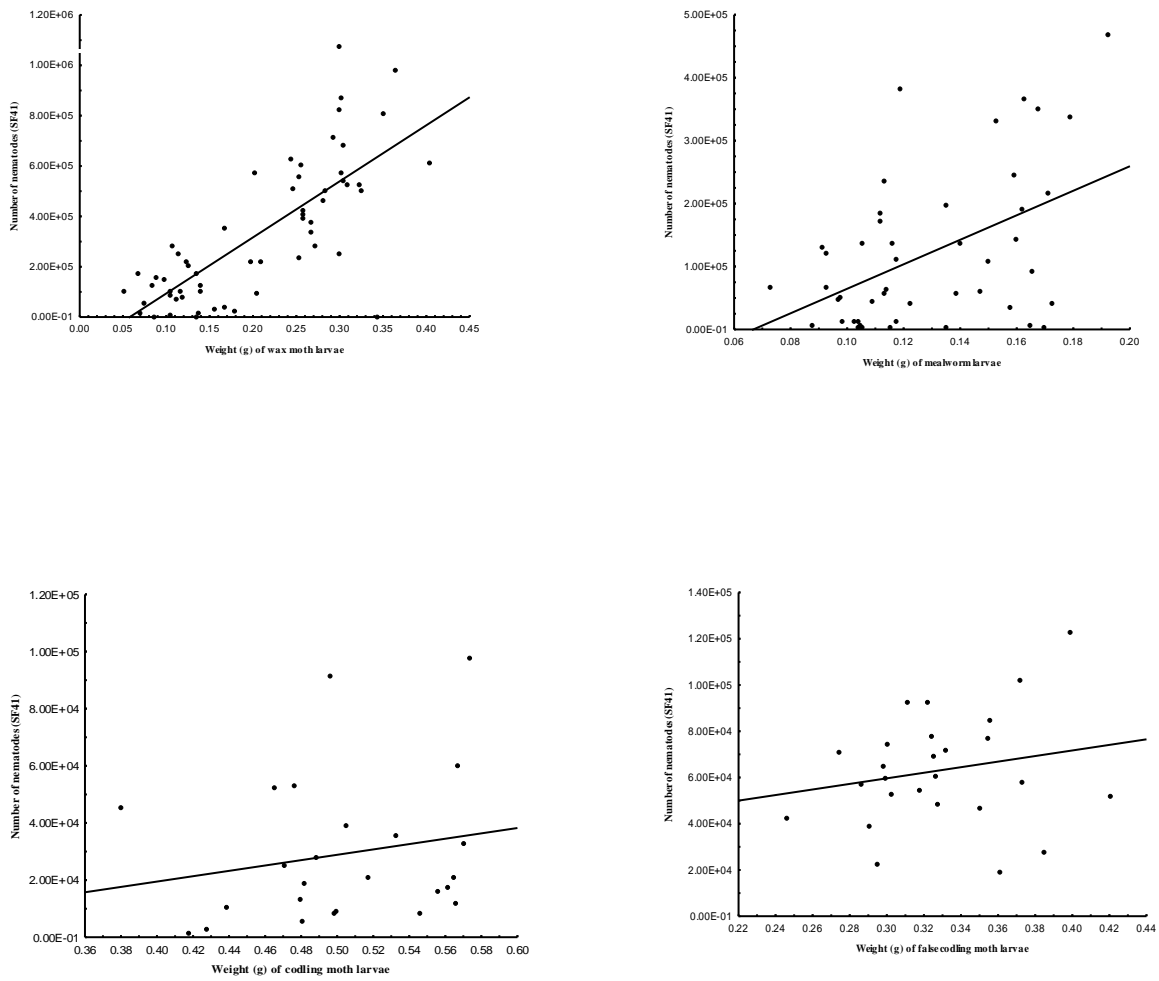


Fig. 6 Scatterplots depicting the correlation between weight (g) of hosts and the number of *H. zealandica* (SF41) progeny produced by each host.

Discussion and conclusion

A review of EPN-related literature with regard to a novel host for the *in vivo* culturing of nematodes presented no suitable alternative hosts to mealworm or to wax moth larvae. Silkworms *Bombyx mori* (L.) were considered, amongst others, as hosts, due to their ability to produce large numbers of nematodes (Zaki et al. 2000; Prabhuraj and Kamar 2003; Kanta et al. 2008). However, obtaining fresh mulberry leaves year round proved to be impractical, and developing an artificial diet for the insects concerned also proved to be both difficult and expensive.

WML and MW, two of the most commonly used insect hosts in nematode culturing (Shapiro-Ilan et al. 2002), were selected for culturing of nematodes in the current study. The insects concerned are highly susceptible to nematodes during the larval stage and are generally inoculated with nematodes when in the last instar (Grewal et al. 1993; Flanders et al. 1996; Boff et al. 2000; Gokte-Narkhedkar et al. 2008). In South Africa, the larvae of these hosts are available from certain pet shops or, in the case of WML, can be collected from infested beehives. Such insect hosts are easy and practical to rear in a non-laboratory environment, since limited resources are required to produce high-quality nematodes (Shapiro-Ilan et al. 2003). In the current study, the larvae of these hosts produced sufficient numbers of nematodes for the *in vivo* culturing process to be economically viable for the small-scale production of local endemic, highly specific and virulent EPN isolates.

Rearing WML is often less expensive compared to rearing insects within the nematode's natural host range (Birah et al. 2008) and the costs associated with rearing MW are often lower than are those associated with WML (Blinova and Ivanova 1987; Gaugler and Han 2002). Diet ingredients of the host diets are also easily available and relatively inexpensive, and the life cycles of the hosts are short enough to ensure a constant and quick supply of larvae during the production process.

A superior holidic diet for both WML and MW was selected according to the ability to produce the heaviest larvae. Diet 1, consisting of wheat flour, wheat bran, milk powder, brewer's yeast, wax powder, honey and glycerol, produced the heaviest larvae. Brewer's yeast and wheat germ has been reported to increase the weight of mature WML (Gross et al. 1996). Brewer's yeast was added to each diet and, in the case of Diet 4, wheat germ was also added. However, the inclusion of milk powder and beeswax were the two ingredients that distinguished Diet 1 from the other diets. Therefore, the probability of the two ingredients included in Diet 1 having an influence on larval weight is high. A study by Flanders et al. (1996) found that the number of IJs produced in large last-instar WML was double that of IJs produced in small last-instar larvae. Selecting a diet that yields adequate numbers of large larvae would thus be advantageous in terms of increasing the number of nematodes produced. The proportion and/or selection of ingredients

in diets plays an important role in the development of larvae, as well as in nematode fitness and quality (Dadd 1963; Lee et al. 2007; Shapiro-Ilan et al. 2008).

The ability of beeswax in WML diets to increase the growth rate of larvae (Beck 1960; Young 1961) was confirmed in the current study by the development of 90% of larvae that hatched in Diet 1 into either pupae or moths prior to the termination of the experiment. Of the larvae reared on Diet 5, only 10% developed into pupae or moths within the same time period of 52 days. The effect of milk powder on larval development has not been well documented, and it is unclear whether the inclusion of the ingredient might have influenced larval weight in the present instance.

Increased insect developmental rate can be influenced by other diet-related parameters besides wax. Diet 4 also produced a high number of WML moths and pupae (88% of hatched larvae) under the same conditions as did Diet 1, despite not containing any wax. Diets rich in carbohydrates, such as Diet 4, have been shown to be able to substitute beeswax as an ingredient, due to the possession of similar nutritional values (Dadd 1966). Accordingly, under the same nutritional and environmental conditions, similar larval development would be expected in diets that are rich in carbohydrates, versus diets containing wax.

Apart from nutritional value, dietary texture can also play an important role in insect larval development. The two diets that produced the heaviest larvae, Diets 1 and 5, were the most compact, moist and clumpy diets. Trapping of metabolic heat within such compact diets could result in increased temperatures at a much higher level compared to the temperature level that would be found in more powdery and dry diets, such as in Diets 2 and 3. Higher temperatures lead to increased larval development (Cymborowski 2000), indicating that dietary texture can, indirectly, have an effect on the developmental rate of larvae. Diets that are dense or high in sugar content, like Diets 1 and 5, also tend to have a lower rate of water loss, due to the accompanying hygroscopic activity of sugars and to the reduction in evaporation caused by denser diet structures (Dadd 1963). The high humidity levels within the diet containers could have been more favourable for larval development, compared to the ambient relative humidity (RH) of 55% in containers of Diets 2 and 3. Suboptimal humidity levels within plastic containers could, thus, have contributed to the low larval weight achieved in Diet 3 and to the drying out of eggs in Diet 2.

Selecting a WML diet, in the present case, can be a trade-off between the cost of the diet and its ability to produce hosts of sufficient weight within a desirable timeframe. Diet 1, which performed best in terms of yielding the heaviest larvae and having the fastest developmental rate, was also the most expensive diet. In comparison, Diets 2 and 3 were the cheapest, but produced larvae of inferior weight.

Surprisingly, Diet 5, consisting of only wheat bran, produced the heaviest MW larvae. The diet concerned was also the cheapest one tested, and would thus be the preferred choice on which to rear MW. Wheat-medium-based diets are the most commonly used diets in mealworm-rearing systems and diets high in carbohydrates have been shown to be necessary for the proper growth and development of the above-mentioned insects (Parween and Begum 2001; Rice and Lambkin 2009). Diet 4 contained a lipid source in the form of sunflower oil. The weight of larvae produced from this diet did not differ significantly from Diet 5 (wheat bran only), which produced the heaviest larvae. Nutritional content, especially lipid-rich diets, have been shown to influence the susceptibility of mealworms to nematode species (Shapiro-Ilan et al. 2008). Testing the infectivity of mealworms reared on various diets was not the aim of this study. However, it could be useful to know the additional effect that lipid-rich diets have on mealworms, especially if they prove to produce larvae of sufficient weight, which was the case with Diet 4.

Significant differences were obtained between insect hosts with regard to final nematode yield. Similar patterns in the nematode production capabilities of various hosts were seen for both nematode isolates used. With both isolates, WML yielded the most nematodes per g host, followed by MW, FCM and CM. Such a findings can possibly be ascribed to the fact that WML contain a high percentage of fat (Finke 2002), and certain lipid components have been shown to play a conducive role in increasing the developmental rate and yield of heterorhabditids (Yang et al. 1997; Yoo et al. 2000; Hatab and Gaugler 2001). The presence of certain lipids in insect diets has also been shown to promote host susceptibility and infection rates (Shapiro-Ilan et al. 2008). The possibility could thus have existed that the specified lipids were present in the WML diet used in the current study, making the WML highly susceptible bioreactors, in which nematodes could proliferate.

Progeny production in WML was the highest, when respectively using *H. zealandica* and *H. bacteriophora*, and differed significantly from the production numbers obtained by other insect hosts. In a study conducted by Flanders et al. (1996), large WML weighing between 0.3 and 0.4 g produced 567 000 *H. bacteriophora* juveniles per host, equalling, on average, 1 620 000 nematodes per g host. The number of juveniles found was less than the number that was attained in the current study. Differences in inoculum concentrations or isolate size of nematodes used in the study by Flanders et al. (1996), compared to those used in the current study, might have been responsible for the lower numbers of nematodes obtained in the other researchers' study. For *H. zealandica*, about 440 000 fewer nematodes were produced in WML compared to what was achieved with using *H. bacteriophora*. The longer IJ body length of *H. zealandica* (685 µm), compared to *H. bacteriophora* (588 µm), could be the determining factor responsible for the lower numbers of *H. zealandica* IJs produced in the same host (Nguyen and Hunt 2007).

Yield obtained using *H. bacteriophora* and MW as host, equaled 42 908 nematodes per insect. In a previous study by Shapiro-Ilan and Gaugler (2002), nematode production in MW larvae was substantially higher, yielding 115 538 *H. bacteriophora* progeny per insect. Differences in the diet of MW during this study and in that of Shapiro-Ilan and Gaugler (2002) could possibly explain the difference in yield. In contrast, using *H. zealandica*, much higher progeny numbers were achieved in MW larvae compared to those achieved when using *H. bacteriophora*. Overall, more *H. zealandica* progeny were produced in all hosts tested, except in WML, where the numbers of *H. bacteriophora* produced were higher. Closer natural association of *H. zealandica* with MW, CM and FCM could have led to higher reproductive potential with such hosts (Shapiro-Ilan and Gaugler 2002).

Since an endemic nematode species, *H. zealandica*, has proven to be highly virulent against CM in South Africa (De Waal, 2008; De Waal et al. 2010, 2011) and a facility for mass-rearing of codling moth had already been established in Stellenbosch, nematode production in the host concerned was also tested. Another insect pest susceptible to the specific nematode isolate is FCM, which is a key pest in the South African citrus industry (Addison 2005). The number of *H. zealandica* IJs produced per g host of CM and FCM larvae were very low compared to the yield achieved per g host for MW and WML. A prominent factor influencing low production in the present case could be the high ratio of surface area to volume of these small hosts, which inherently leads to the availability of fewer nutrients to the feeding nematodes per g host. Nematodes can, however, easily adapt to the host in which they are continuously cultivated. It would, therefore, be beneficial to use insect pests like CM or FCM as rearing hosts periodically for a few generations, in order to prevent host adaptation and to maintain virulence of the particular EPN species specified (Grewal et al. 1993).

Freezing of insect hosts could be an effective and practical way of storing hosts that prove to produce competitive numbers of nematodes. During the current study, WML and MW were frozen for 72 h prior to inoculation. *Heterorhabditis bacteriophora* progeny production from frozen WML was very low compared to the number of nematodes produced within fresh WML. Initially, high contamination levels were experienced, which was assumed to be the cause of the low production numbers obtained, and, consequently, 0.1% formaldehyde was added to the nematode suspension and applied to each WML. Even though less contamination occurred, a low yield was still achieved in frozen WML. In a trial conducted by San-Blas (2009), only 30% of WML were colonised by *H. bacteriophora* when inoculated after being killed by freezing for 96 h. In addition to that, the presence of *H. bacteriophora* IJs in WML cadavers that were inoculated 48 h and 72 h after freezing differed significantly ($p < 0.05$). They found a lower number of *H. bacteriophora* IJs to be present in the older cadavers that had been dead for 72 h. Possible reasons for the lower production numbers achieved in the present study could be due to the inability for

successful nematode development to take place inside host cadavers that had been dead for 72 h, due to unfavourable conditions inside the hosts. Heterorhadtids have also been shown to be less disposed to scavenging behaviour compared to Steinernematidits, and to have a lower penetration rate. Such factors could have contributed to the lower yield obtained in the present instance. A shorter period between freezing and inoculation of hosts could overcome the problem of unfavourable conditions inside hosts, but would defy the main purpose of freezing hosts as a way to store them.

Frozen MW, in contrast, produced almost similar numbers of *H. bacteriophora* progeny as did its fresh counterpart. Lower contamination levels observed in MW cadavers could be one of the reasons for the better nematode production levels achieved. If MW larvae are thus selected as a suitable host, freezing could be a viable option of storage.

One of the objectives of the current study was to determine whether a positive linear relationship exists between weight of larvae and the number of nematodes produced. The significant linear relationship that existed between WML and MW and both nematode species, respectively, is in agreement with studies done by Dutky et al. (1964), Blinova and Ivanova (1987) and Flanders et al. (1996). The latter authors reported that a linear relationship existed between the size of host larvae and number of nematodes produced, but was inversely proportional to yield per mg host. However, at some point the number of nematodes produced can decrease, even though host weight increases, possibly as a result of an increase in competition for available resources (Selvan et al. 1993). Determination of the number of hosts needed to produce a specific number of nematodes to treat an area infested with a key insect pest could be easily calculated using the respective regression formulae obtained in the current study, should WML and MW be used as hosts.

In the case of frozen MW larvae, a negative correlation occurred for both EPN species used in this study, although it was not significant. Contamination could, however, have had an influence on the results obtained. No significant linear relationship occurred between nematode production and the weight of CM or FCM larvae. However, variation in CM and FCM quality, as a result of not being produced onsite could have caused inconsistent numbers of nematodes to be produced.

To summarise, either WML or MW could be used as nematode hosts for the two nematode species, *H. bacteriophora* (SF351) and *H. zealandica* (SF41), based on the large numbers that they yield. The yield obtained from these hosts can also easily be determined, due to the linear correlation between weight of the larvae and the number of nematodes produced. Ultimately, Diet 1 is recommended for WML culturing, if it proves to be an economically viable option, while Diet 5, being both cost-efficient and producing the heaviest larvae, is the recommended diet for MW rearing.

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Chapter 4

Optimisation of inoculation techniques for *in vivo* mass culture of entomopathogenic nematodes through nematode and insect host manipulation

Abstract

The inoculation of host insects with nematodes is a critical step in the *in vivo* production of entomopathogenic nematodes. The *in vivo* production process can only be efficient if each of the steps in the process is optimised. Obtaining high and consistent infection rates of insect hosts during the inoculation process is one of the components that contribute to enhancing EPN production efficiency. To improve host susceptibility and nematode infectivity, the inoculation process can be manipulated by using physical and chemical stressor methods. Three different inoculation treatments were compared, using *Galleria mellonella* (L.) (wax moth larvae) and *Tenebrio molitor* (L.) (mealworms) as hosts, and *Heterorhabditis bacteriophora* and *H. zealandica* as the respective inoculum sources. The highest percentage infection of both insect hosts was achieved by pipetting nematodes onto hosts, followed by immersion of hosts into nematode suspensions, and, lastly, by shaking hosts together with nematode inoculum. It was found that the effects of stressor treatments on mealworm larvae did not improve infection rates of *H. bacteriophora* and *H. zealandica*. Mealworm larvae were exposed to 70°C tap water in an attempt to compromise their defence mechanisms prior to inoculation. However, infection levels of 11.50% ± 2.44% for *H. bacteriophora* and 20.50% ± 3.87% for *H. Zealandica* obtained were lower than that of the control. Furthermore, pre-inoculation treatment of infective juveniles with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$, did not significantly increase infection rates, compared to the control. Mealworm infection was 81.00% ± 3.62% for treated *H. zealandica* in comparison to 80.00% ± 3.40 for the control. Using treated *H. bacteriophora*, infection levels of mealworms were 47.00% ± 3.85%, compared to the control at 48.00% ± 4.85%. Host and nematode stressor methods tested did not lead to an improvement in infection levels for the specific hosts and nematode species used, and would therefore not contribute to the *in vivo* production process.

Introduction

Entomopathogenic nematodes (EPNs) are endoparasitic organisms that possess desirable pest control attributes and that are effective biological control agents against several soil-borne and foliar pests. EPNs can be produced in large numbers by *in vivo* or *in vitro* culturing techniques (Gaugler and Han 2002; Shapiro-Ilan and Gaugler 2002). *In vivo* production is a cost-efficient method of producing nematodes on a smaller scale. The *in vivo* culturing process can be split up into five steps: host and nematode selection; inoculation of hosts; harvesting of nematodes; formulation of nematodes; and application in the target area. The efficacy of host inoculation with EPNs greatly influences the nematode yield achieved (Shapiro-Ilan and Gaugler 2002). Efficient *in vivo* production depends on a high level of host infection during inoculation, and even more so with the scaling-up of production (Gaugler 2002). Low levels of infection lead to the time-consuming and expensive task of removing naturally dead hosts to avoid further contamination of other insect hosts (Woodring and Kaya 1988). To ensure maximal infections, parameters to optimise inoculating techniques should be implemented in the production process (Lacey and Brooks 1997; Gaugler 2002; Shapiro-Ilan et al. 2002).

The efficiency of inoculation techniques can be influenced by host density, nematode concentration, and the inoculation method used (Shapiro-Ilan and Gaugler 2002; Shapiro-Ilan et al. 2002). Apart from such factors, optimally suited environmental conditions during inoculation, as well as a close natural association between the host and the nematode, further improve chances of high infectivity levels (Shapiro-Ilan et al. 2004). Inoculation methods that have been applied and tested in prior studies are: immersion; spraying; pipetting; and application of nematodes directly to the food source of target insects (Blinova and Ivanova 1987; Shapiro-Ilan and Gaugler 2002; Shapiro-Ilan et al. 2002). It has been demonstrated that significant differences can occur among inoculation methods used, with regard to time-efficiency and latent infections obtained (Blinova and Ivanova 1987; Flanders et al. 1996; Gaugler et al. 2002; Shapiro-Ilan et al. 2002).

Additional measures that can be applied to optimise latent infection levels are through applying physical and chemical stress to insect hosts and nematodes (Brown et al. 2006). Doing so could enhance susceptibility of hosts by compromising insect host defenses. The measure could be beneficial when rearing costs of a highly susceptible host are more expensive compared to those of a host that is less susceptible to the specific nematode species (Gaugler and Han 2002). For instance, rearing of mealworm larvae (MW) has proven to be less expensive than has rearing of wax moth larvae (WML) (Brown et al. 2006), but MW are also less susceptible, compared to WML, to certain EPN strains used for commercial pest control strategies. Therefore, if imposing physical and chemical stress upon MW proves to enhance

infection to acceptable levels, *in vivo* production process costs can be reduced when MW are used. Prior studies have proved that physical stress in the form of sublethal heat treatments, by immersing hosts in hot water (50-80°C), or exposing them for periods of time to dry heat (35-40°C), can also increase the susceptibility of host insects (Brown et al. 2006). In studies by Jaworska et al. (1997a, b) and Brown et al. (2006), the effect of chemical stress on nematode infectivity, applied by mixing nematodes with a solution of manganese and magnesium ions prior to being used for inoculation, was tested. Their findings suggested that submersion of MW into a $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ / nematode solution increased the MW mortality and infection levels, as a consequence of increased nematode virulence (Brown et al. 2006; Jaworska et al. 1997a, b).

The objective of the current study was to test different methods of nematode and insect host manipulation, which would lead to the optimisation of host infection levels during the *in vivo* mass culture of nematodes. Three nematode inoculation techniques were compared, as well as the efficacy of three treatments used to improve nematode infectivity through host and nematode manipulation. Two locally selected, endemic South African nematode strains of two different species of EPNs were used.

Materials and methods

Source of nematodes and host insects

Heterorhabditis bacteriophora Poinar, 1967 (SF351) (Genbank accession number EU699436) and *H. zealandica* Poinar, 1990 (SF41) (Genbank accession number FJ455843) from the Stellenbosch University nematode collection (Malan et al. 2006) were used. Infective juveniles (IJs) were produced by *in vivo* culturing in WML. The *in vivo* production process was based on the modified White trap method (White 1927; Dutky et al. 1964; Woodring and Kaya 1988; Lindegren et al. 1993). IJs were harvested for up to a week post emergence and stored horizontally in the dark at 14°C in 50 ml distilled water, in 160-ml ventilated culture flasks. Culture flasks were shaken on a weekly basis to facilitate aeration. IJs used in all experiments conducted were not older than four weeks.

WML and MW were obtained from laboratory cultures. WML were cultured in ventilated plastic containers on a diet consisting of 118 g wheat flour, 206 g wheat bran, 118 g milk powder, 88 g brewer's yeast, 24 g wax powder, 175 ml honey and 175 ml glycerol (Chapter 2). MW larvae were reared in wooden culture boxes on 100% wheat bran and, as an added water source, carrots were used (Chapter 2). Both cultures were reared at 25°C in the dark, as was described in

Chapter 2. Last-instar larvae of both insects were used in all experiments.

Post-inoculation protocol

A total of 13 Petri dishes with 10 insect larvae each ($n = 130$ larvae) for the three different inoculation techniques and for each host and nematode species combination were used. After inoculation, Petri dishes, containing inoculated hosts for the different treatments, were placed in sealed plastic containers ($11 \times 11 \times 7.5$ cm), fitted with moist paper towels and incubated at 25°C . After two days, the percentage mortality of the insect hosts was recorded for each treatment. Mortality was defined by lack of host movement on prodding with a pair of tweezers. Percentage host infection of each treatment was recorded after seven days, based on colour change. Infection was confirmed by means of dissection of the host with the aid of a dissecting microscope.

Inoculation by pipetting

Inoculation of insect hosts by pipetting involved placing 10 MW or WML in a 90-mm Petri dish lined with filter paper (Whatman No. 1). Each Petri dish was inoculated with 500 μl tap water containing IJs at a concentration of 200 IJs / 50 μl of either *H. zealandica* or *H. bacteriophora*, by means of an Eppendorf micropipette. After inoculation, the post-inoculation protocol was followed. The experiment was repeated on a different test date.

Inoculation by shaking

For the ‘inoculate-and-shake’ method, 130 insect larvae of either WML or MW were placed into one small plastic container (250 ml), to which 1 ml nematode inoculum, containing 26 000 IJs (200 IJs per host) of either *H. zealandica* or *H. bacteriophora* was added (Fig. 1A). The container was sealed and shaken by hand for one minute, to ensure coverage of the surface area of the insect bodies with nematodes. Insects were then removed from the container using a pair of tweezers, and 10 insects were placed into a 90-mm filter-paper-lined Petri dish. After inoculation, the post-inoculation protocol was followed and the experiment was repeated on a different test date.

Inoculation by immersion

The immersion technique involved placing 130 mealworms in a small tea sieve (1200 μm) and attaching a plastic lid to the top of the sieve, to avoid mealworms floating out of the sieve when submerged. The sieve was then dipped for five seconds into a 500-ml plastic container, containing a 300-ml suspension of either *H. zealandica* or *H. bacteriophora*, at a concentration of 200 IJs / 50 μl (Fig. 1B). The sieve was then placed for two seconds on a single sheet of paper towel, to allow absorption of excess suspension. Insect hosts were picked from the sieve with a pair of tweezers and ten hosts were placed into individual 90-mm Petri dishes, lined with moist filter paper, whereafter the post-inoculation protocol was followed. The experiment was repeated on a different test date.



Fig. 1 (A) Plastic container used for the ‘inoculate-and-shake’ method, containing 130 wax moth larvae, and (B) sieve containing 130 mealworm larvae suspended above a 300-ml suspension of nematodes.

Post-stress treatment protocol

After each of the stress treatments, 10 MW were placed in each of the 10 filter-paper-lined Petri dishes (90-mm diameter) and incubated at 25°C in the dark in sealed plastic containers (11 × 11 × 7.5 cm), fitted with a moist paper towel. Percentage mortality of MW for each treatment was recorded after two days. Percentage infection was recorded after seven days. Infection was evaluated, based on cadaver colour change. EPN infection was confirmed by dissection of the host larva with the aid of a dissecting microscope. The same number of MW were immersed in a suspension that contained only either *H. zealandica* or *H. bacteriophora*, and served as the untreated control.

Warm-water treatment

Treating the host with hot tap water involved the immersion of 100 MW in a sieve (1200 µm) in 300 ml of 65-70°C warm water for five seconds. The sieve was momentarily dried of excess water for two seconds by blotting it on a single paper-towel sheet, before immersing it for another five seconds into a beaker containing 250 ml of a nematode suspension containing either *H. zealandica* or *H. bacteriophora* at a concentration of 200 IJs / 50 µl. Afterwards, the post-stress treatment protocol was followed. The experiment was repeated on a different test date.

Manganese $Mn^{2+}SO_4.H_2O$ treatment

Treating the IJs of the two respective EPN species with manganese involved adding 1.7mM $Mn^{2+}SO_4.H_2O$ to a 250-ml nematode suspension, at a concentration of 200 IJs / 50 µl. MW, contained in a sieve, were then immersed in the nematode/ $Mn^{2+}SO_4.H_2O$ suspension for five seconds and placed on a paper towel for two seconds. Afterwards, the post-stress treatment protocol was followed. The experiment was repeated on a different test date.

Combination of hot water and $Mn^{2+}SO_4.H_2O$ treatment

The last treatment was a combination of the first and second treatments, in which 100 MW were immersed in 65-70°C warm tap water for five seconds, placed on a paper towel for two seconds and then immersed into the nematode/ $Mn^{2+}SO_4.H_2O$ suspension for five seconds. Afterwards, the post-stress treatment protocol was followed and the experiment was repeated on a different test date.

Data analysis

The data analysis software system Statistica version 10 (Statsoft Inc. 2011) was used to perform all statistical analysis. Percentage insect host mortality and EPN infection data were analysed by using a one-way ANOVA with post-hoc comparisons of means, using Bonferroni's method. For each experiment, data from both trials were pooled if no interaction was observed between trials. In cases where there was significant interaction among trials, the data of each trial were analysed separately.

Results

Effects of the three inoculation methods on mortality and infection of WML, using H. bacteriophora (SF351)

Significant interaction was observed between the initial (Trial 1) and repeat (Trial 2) trials, due to difference in percentage infectivity levels ($p < 0.001$), and was thus analysed separately. The average mortality rate of WML for all inoculating treatments tested was above 80%. The mean percentage mortality achieved for the three inoculating treatments differed significantly among each other on both trial dates (Trial 1: $F_{(2, 36)} = 7.511$, $p < 0.05$; Trial 2: $F_{(2, 36)} = 12.484$, $p < 0.001$). The highest percentage insect mortality obtained in both trials was with pipetting. Inoculating using a pipette resulted in $93.84\% \pm 1.80\%$ (mean \pm SE) mortality in Trial 1 and in 100% mortality in Trial 2. Using the ‘inoculate-and-shake’ treatment in Trial 1 resulted in a WML mortality of 100%; however, in Trial 2 it declined to $74.61\% \pm 7.04\%$. The mean percentage mortality recorded using immersion as inoculating treatment resulted in an $88.46 \pm 3.17\%$ mortality for Trial 1 and in an $99.23\% \pm 0.80\%$ mortality for Trial 2 (Figs. 2 and 3).

Notable differences in infection rates of WML with *H. bacteriophora* among treatments was recorded for Trial 2, but not for Trial 1 (Trial 1: $F_{(2, 36)} = 0.256$, $p = 0.775$; Trial 2: $F_{(2, 34)} = 310.330$, $p < 0.001$). The lowest recorded infection rate was during Trial 2, using immersion as inoculating treatment ($9.17\% \pm 2.60\%$). Contrary to that, the mean percentage infection recorded for the same treatment during Trial 1 was $90\% \pm 2.77\%$. The highest infection rates were obtained with pipetting in both trials (Trial 1: $92.31\% \pm 1.66\%$; Trial 2: $96.92\% \pm 2.37\%$). Relatively consistent infection rates between trials were obtained using the ‘inoculate-and-shake’ treatment with $90.77\% \pm 2.40\%$ infection recorded in Trial 1 and $85.83\% \pm 3.13\%$ in Trial 2 (Figs. 2 and 3).

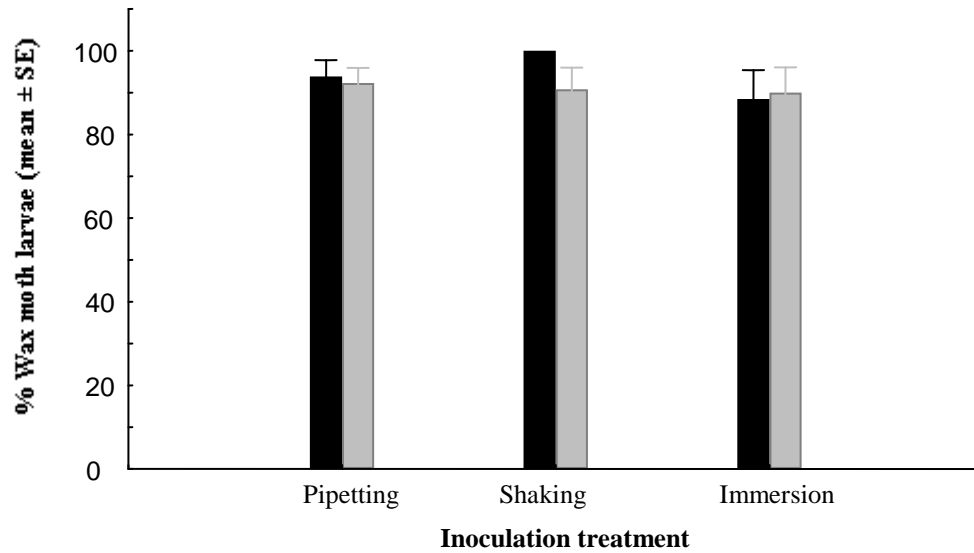


Fig. 2 Trial 1 for the mean \pm SE (standard error) (95% confidence interval) wax moth larval mortality (■) and infection (□) for each of the three inoculating treatments, using *H. bacteriophora* (SF351).

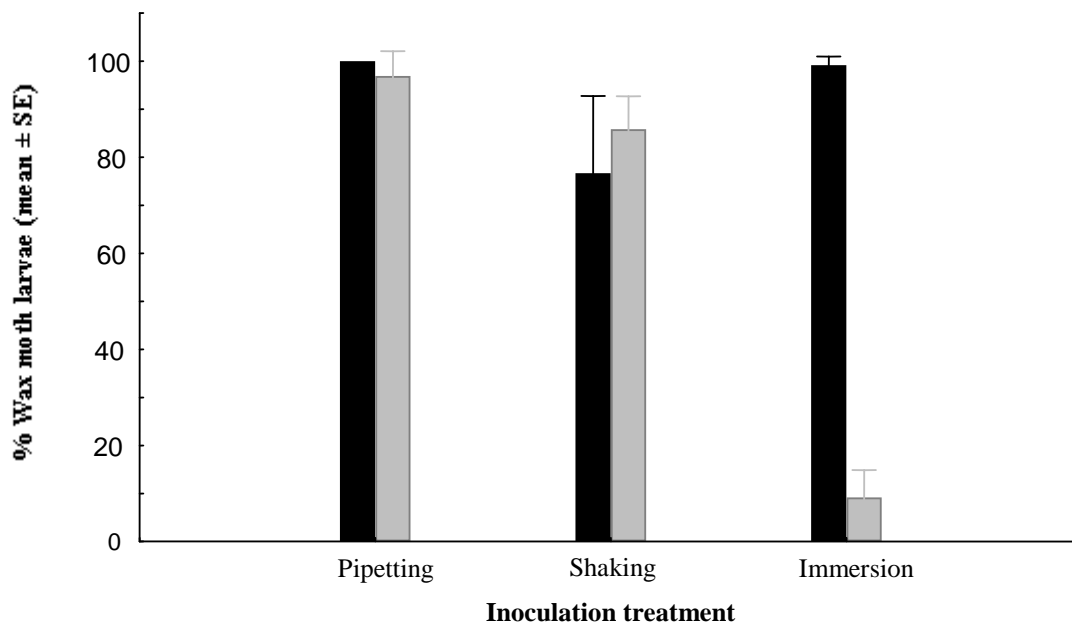


Fig. 3 Trial 2 for the mean \pm SE (95% confidence interval) wax moth larval mortality (■) and infection (□) for each of the three inoculating treatments, using *H. bacteriophora* (SF351).

Effects of three inoculation methods on mortality and infection of WML, using H. zealandica (SF41)

No significant interaction was observed between the results of the two trials, and the data were thus pooled before analysis. Significant differences occurred among treatments regarding mortality rates ($F_{(2, 75)} = 6.0$, $p < 0.05$) and infection rates ($F_{(2, 75)} = 8203.65$, $p < 0.001$). The highest mortality of WML and infection rates for *H. zealandica* were obtained with pipetting (100% mortality and 99.23% \pm 0.53% infection). An extremely low infectivity rate (3.08% \pm 0.92%) was achieved with ‘inoculate-and-shake’, even though the mortality rate was 100%. Mortality rates of 98.08% \pm 0.79% and infection rates of 100% were recorded using immersion as inoculating treatment (Fig. 4).

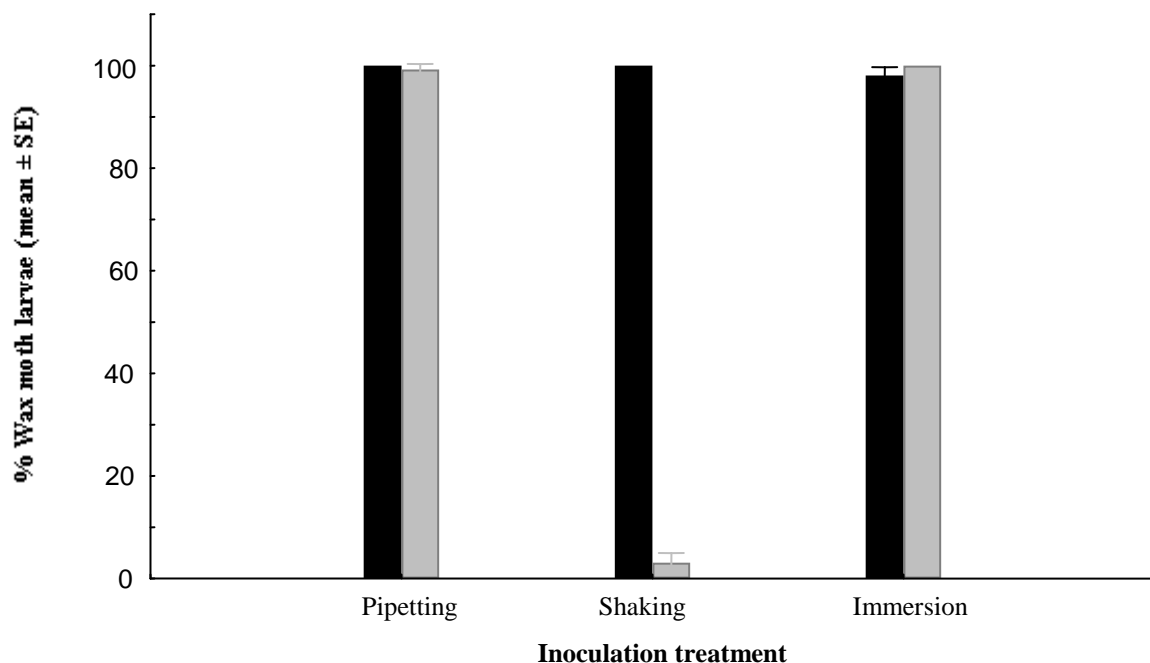


Fig. 4 The mean \pm SE (95% confidence interval) wax moth larval mortality (■) and infection (□) for each of the three inoculating treatments, using *H. zealandica* (SF41).

Effects of three inoculation methods on mortality and infection of MW, using H. bacteriophora (SF351)

No significant interaction was observed between the results of the two trial dates, and the data were thus pooled before analysis. Inoculating treatments differed significantly from one another, with regard to percentage mortality ($F_{(2, 72)} = 71.324$, $p < 0.001$) and percentage infection ($F_{(2, 69)} = 107.63$, $p < 0.001$) recorded. Mortality of MW obtained with *H. bacteriophora* for pipetting was $82.69\% \pm 3.62\%$, followed by $56\% \pm 4.24\%$, achieved with immersion. The lowest mortality rate was recorded for 'inoculate-and-shake', at $48.46\% \pm 5.84\%$. A similar and consistent trend was observed for the infection rates compared to the mortality rates, with pipetting achieving the highest infection rates at $76.15\% \pm 2.83\%$, followed by immersion ($74.80\% \pm 3.32\%$) and 'inoculate-and-shake' ($24.23\% \pm 2.43\%$) (Fig. 5).

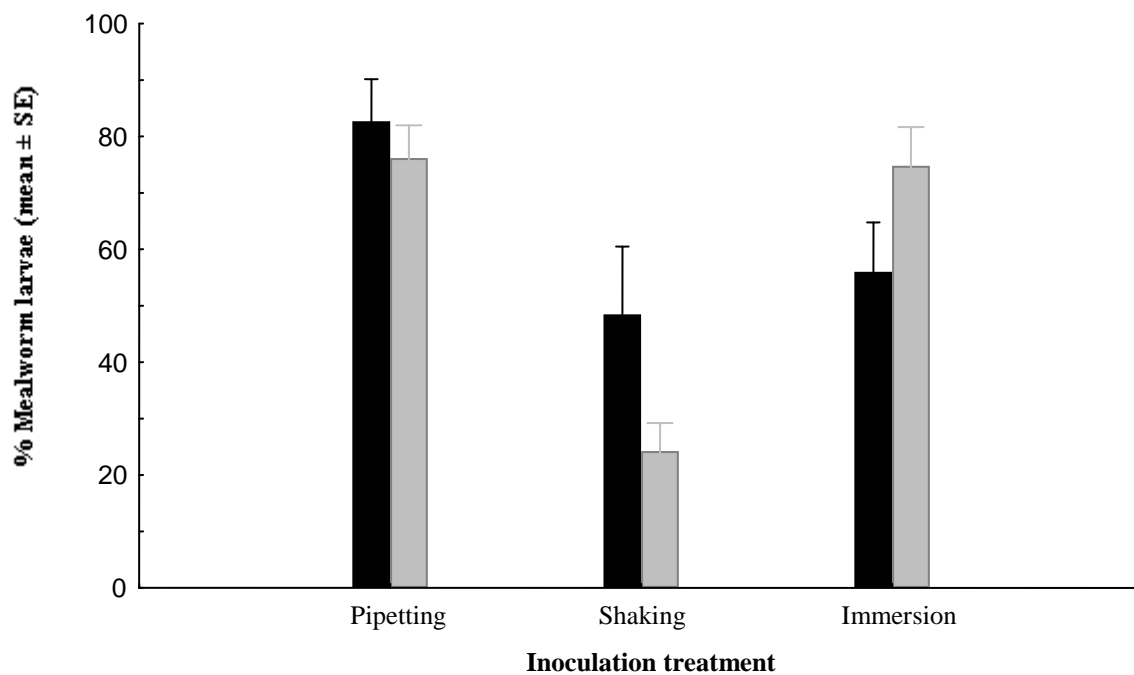


Fig. 5 The mean \pm SE (95% confidence interval) mealworm larval mortality (■) and infection (□) for each of the three inoculating treatments, using *H. bacteriophora* (SF351).

Effects of three inoculation methods on mortality and infection of MW, using H. zealandica (SF41)

There was significant interaction between the two trials conducted ($p < 0.001$). Significant differences occurred between treatments with *H. zealandica* for mortality of MW in both trials (Trial 1: $F_{(2, 36)} = 222.28$, $p < 0.001$. Trial 2: $F_{(2, 36)} = 109.29$, $p < 0.001$), as well as for infection (Trial 1: $F_{(2, 36)} = 382.22$, $p < 0.001$. Trial 2: $F_{(2, 36)} = 946.79$, $p < 0.001$). Pipetting, in both trials, was the most efficient inoculating treatment, with $96.92\% \pm 1.75\%$ and 100% infection and $89.23\% \pm 3.09\%$ and $93.85\% \pm 1.80\%$ mortality obtained in the respective trials. Even though a high mortality rate (100%) was observed in trial 2 for the ‘inoculate-and-shake’ treatment, no insect larvae was infected using the treatment concerned, making it the least effective treatment of all treatments employed. Mortality and infection in both trials for immersion were within the same range, and competitive infection rates were obtained using the method specified. In trials 1 and 2, the mortality rates were $61.54\% \pm 3.90\%$ and $53.08\% \pm 3.82\%$, respectively. Infection rates were slightly higher compared to mortality rates, at $72.31\% \pm 4.11\%$ and $78.46\% \pm 2.96\%$ for trials 1 and 2, respectively (Figs. 6 and 7).

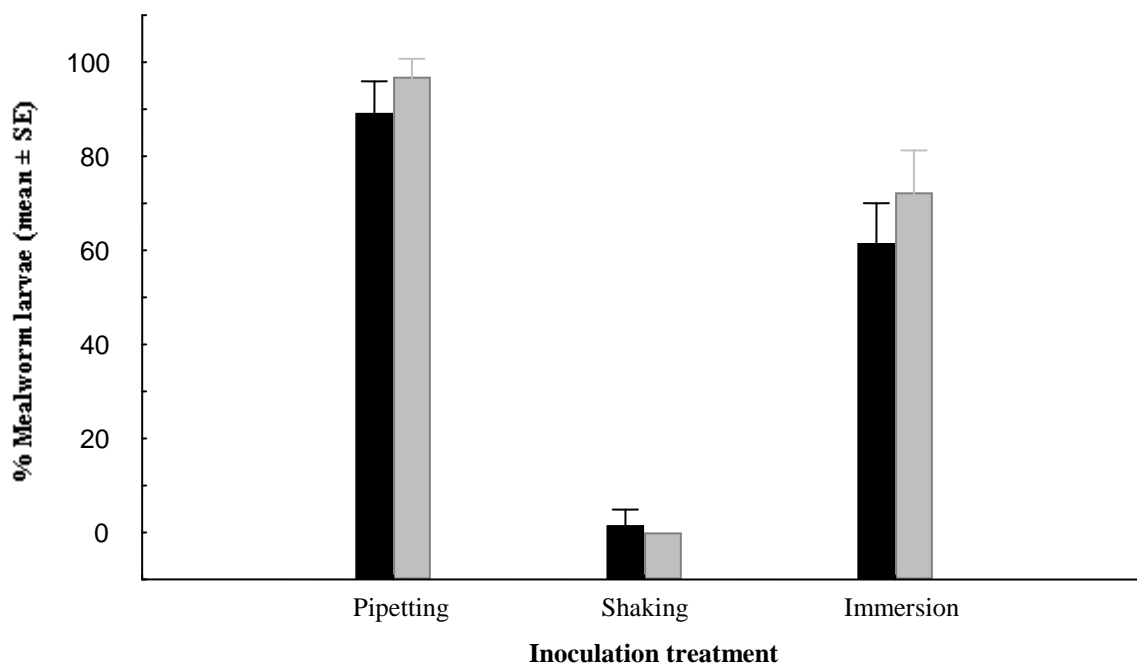


Fig. 6 Trial 1 for the mean \pm SE (95% confidence interval) mealworm larval mortality (■) and infection (▒) for each of the three inoculating treatments, using *H. zealandica* (SF41).

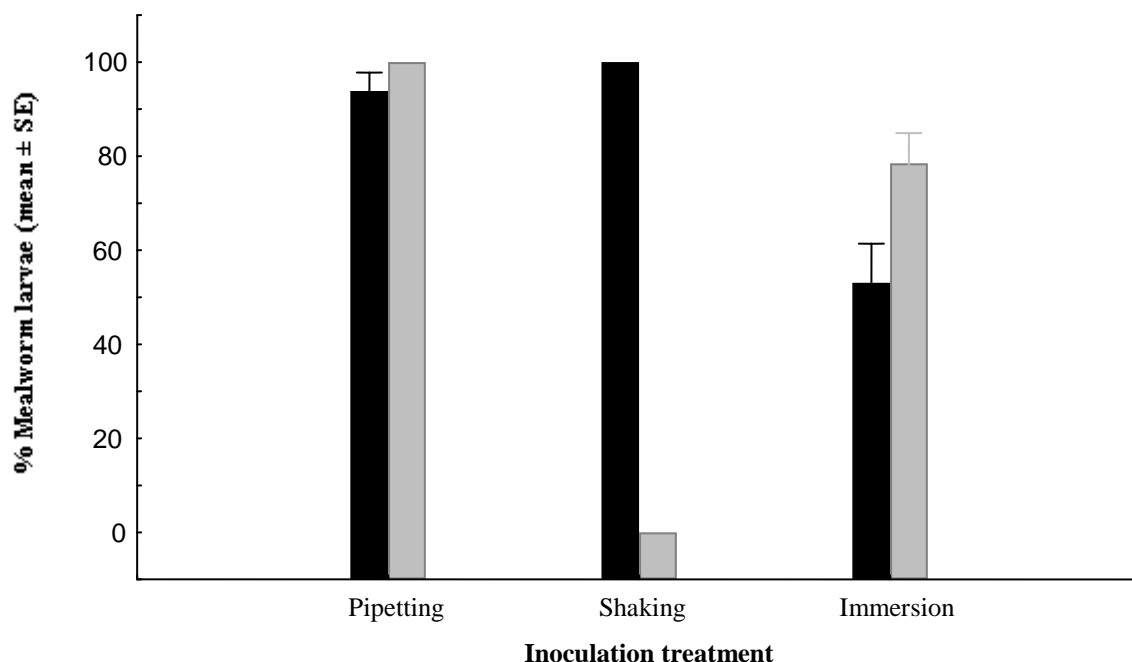


Fig. 7 Trial 2 for the mean \pm SE (95% confidence interval) mealworm larval mortality (■) and infection (■) for each of the three inoculating treatments, using *H. zealandica* (SF41).

Effects of physical and chemical stress methods on mortality and infection of MW, using H. bacteriophora (SF351)

There was a significant interaction between treatments regarding both MW mortality ($F_{(3, 76)} = 314.448$, $p < 0.001$) and infection ($F_{(3, 76)} = 33.687$, $p < 0.001$). The two treatments, in which MW larvae were immersed into hot water, led to 100% mortality of larvae. Only $14.50\% \pm 4.44\%$ mortality was recorded for the control treatment, in which larvae were immersed into a nematode-water suspension. A slight increase of 1.5% in mortality rate was achieved by immersing hosts into a suspension containing both $Mn^{2+}SO_4 \cdot H_2O$ and nematodes.

Even though the mortality rate was initially low for the control treatment, infection rates were the highest, at $48\% \pm 4.85\%$, compared to the other treatments. MW infection by *H. bacteriophora* recorded for the $Mn^{2+}SO_4 \cdot H_2O$ treatment followed closely, at $47.00\% \pm 3.85\%$. An infection rate of $12.50\% \pm 2.39\%$ was obtained by pre-treating MW larvae and nematodes with both $70^\circ C$ water and $Mn^{2+}SO_4 \cdot H_2O$. The lowest infection rate of $11.50\% \pm 2.44\%$ was recorded when MW larvae were treated with only the $70^\circ C$ water treatment (Fig. 8).

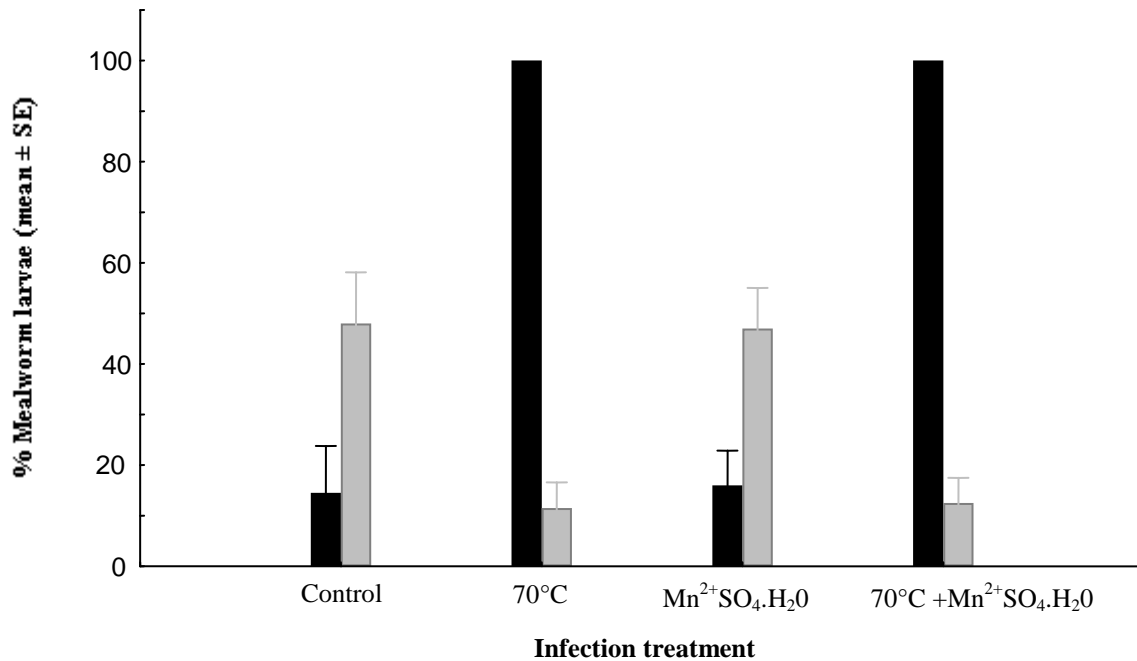


Fig. 8 The mean \pm SE (95% confidence interval) mealworm larval mortality (■) and infection (▒) for each of the three applied physical and stress methods, using *H. bacteriophora* (SF351).

Effects of physical and chemical stress methods on mortality and infection of MW, using H. zealandica (SF41)

Significant differences occurred between treatments regarding MW mortality ($F_{(3, 76)} = 67.521$, $p < 0.001$) and infection ($F_{(3, 76)} = 123.185$, $p < 0.001$). The highest mortality rate of MW (100%) for *H. zealandica* was recorded for the 70°C water treatment of MW larvae and 70°C water plus $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ treatments. That was followed by $58.50\% \pm 5.34\%$ mortality, achieved with the $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ method. The control produced the lowest MW mortality rate at $47.50\% \pm 4.03\%$.

Latent infection rates were the lowest with treatments where MW larvae were pre-treated using warm water, even though most larvae died with the application of the physical stress measure concerned. Only $9.00\% \pm 2.80\%$ of MW larvae were infected applying the 70°C water plus $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ treatment, and $20.50\% \pm 3.87\%$ using the 70°C water treatment alone. Treating nematodes with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ improved infection rates slightly, achieving 1% higher infection rates compared to the rates attained with the control. With 81.00% MW infection achieved, treating nematodes with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ was found to be the most effective treatment (Fig. 9).

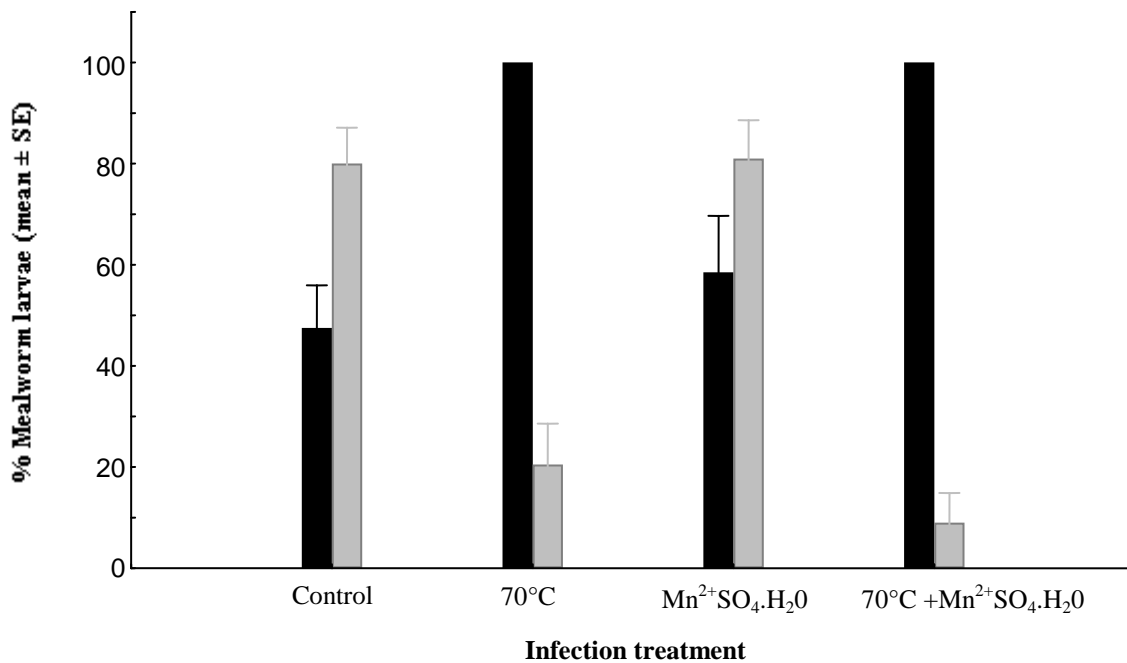


Fig. 9 The mean ± SE (95% confidence interval) mealworm larval mortality (■) and infection (□) for each of the three applied physical and stress methods, using *H. zealandica* (SF41).

Discussion and conclusion

Optimising infection levels during inoculation is a critical step in the development of an effective and cost-efficient *in vivo* nematode production process (Shapiro-Ilan et al. 2004). In previous studies, Jaworska et al. (1997a, b; 1999) and Brown et al. (2006) also used various techniques to increase the infection of insect larvae, by applying physical and chemical stress to hosts and nematodes. The two nematode species, *H. bacteriophora* and *H. zealandica*, that were used during this part of the study, are local strains that have been selected to provide satisfactory control of mealybug (Stokwe 2009), codling moth (De Waal 2008; 2011), false codling moth (Malan et al. 2011) and the banded fruit weevil (Ferreira 2010) on citrus and deciduous fruits in the South African agriculture industry. The hosts used in this study were commonly available, and easy and cheap to culture.

Differences in percentage mortality and infection of both WML and MW occurred among some of the trials, despite attempts to standardise the inoculation procedures. Significant interactions occurred between trials in which WML were inoculated with *H. bacteriophora* and MW with *H. zealandica*. The interaction occurring between the trials can possibly be due to virulence variation within batches of the respective nematode species. According to Griffin and Downes (1994), variation in *H. bacteriophora* batches is especially common. In addition, the general condition of the

host may have influenced mortality and infectivity. Different host sizes and recency of last feeding tend to influence host susceptibility to nematodes (Kondo 1987; Flanders et al. 1996; Boff et al. 2000).

Both percentage mortality and percentage infection was recorded for each treatment. However, from the results obtained, it was concluded that percentage insect mortality cannot be used as an indication for the percentage infection, as significant variation can occur between the two parameters concerned. For example, application of the ‘inoculate-and-shake’ treatment to MW larvae led to a mortality rate of 49%, two days after inoculation with *H. bacteriophora*. However, the mean percentage infection after seven days was almost half, at 24%. Determining infection is labour-intensive, as hosts need to be washed and individually dissected, in order to observe developing nematodes in the haemocoel by means of a microscope. The higher mortality rate can be due to several external factors that are able to cause the natural death of the insect host. Apart from biotic and abiotic factors (Woodring and Kaya 1988; Grewal et al. 1994; Flanders et al. 1996; Shapiro-Ilan et al. 2002), physical damage of the hosts during experimental handling can also cause death of the host insect. Efficiency of methods for the optimisation of inoculation for *in vivo* production purposes should therefore be measured according to the levels of latent infections and not of mortality (Shapiro and Lewis 1999). Latent infections lead to the production of nematodes; therefore, nematode yield is directly influenced by the number of latent infections obtained (Shapiro-Ilan and Gaugler 2002).

The general procedure used for inoculating insects in the laboratory is pipetting. It also proved to be the most effective inoculating method for most treatment combinations investigated during the current study, excluding the *H. zealandica* and WML combination, in which immersion proved to produce higher infection rates. An infection rate of above 90% was maintained for all nematode–host combinations, except for MW inoculation with *H. bacteriophora*, where infection dropped to 76%. This lower infection rate could possibly have been as a result of a suboptimal nematode–host combination, compared to the other combinations. Even though pipetting produced the highest infection rates, it is impractical when nematodes are produced on a larger scale, due to its time-consuming nature (Shapiro-Ilan et al. 2002). Therefore, more suitable, alternative inoculating techniques were considered, such as the immersion of WML in an *H. zealandica* suspension, which resulted in a 100% infection. High infection rates (90%) of WML in trial 1 were also experienced with immersion, when *H. bacteriophora* was used. In contrast, the second trial produced very low levels of WML infection when immersion was used as the inoculation method. It is unclear what was specifically responsible for the result obtained, but the use of substandard-quality inoculum can possibly be due to the phenomena concerned. To obtain conclusive results, the same inoculation technique should be repeated with different batches of nematodes. Immersion of WML could be a more practical and time-efficient method of inoculation.

Infection rates obtained by applying the immersion technique to inoculate MW were much lower compared to pipetting, yet still offered the best alternative. When *H. bacteriophora* was used as the inoculum with MW, 75% infection was achieved. Infection levels within the same range were obtained when *H. zealandica* was used, where the mean percentage infection for trials 1 and 2 were, respectively, 72% and 79%. The success of the treatment was surprising, since MW are known to have an extremely smooth cuticle covering the small surface area of the body, making it difficult for nematodes to adhere to the host when they are immersed for a short period of time into a suspension (Shapiro-Ilan et al. 2002). It seems, however, that immersing MW for five seconds in a nematode suspension provided sufficient time for the nematodes to attach and adhere to the insect larva. Despite immersion being more time-efficient and delivering competitive levels of infection, it should be noted that more nematodes are required for the treatment specified, compared to the number required for the other treatments (Shapiro-Ilan et al. 2004). Shapiro-Ilan et al. (2002, 2004) also state that it appears that the immersion of MW is not a viable method for the mass production of EPNs, if infection levels of 90% and higher are required, which is said to be the standard acceptable percentage for mass-producing nematodes. The ‘inoculate-and-shake’ method performed very poorly using MW and both nematode strains respectively, with infection levels being below 25%. It is, thus, concluded that said method is not an effective inoculation method for MW.

MW has a smooth cuticle and a smaller surface area, compared to WML. Such morphological characteristics of the two respective insect hosts could impede nematode infection, making mealworms less susceptible to the nematodes. Therefore, to increase the usefulness of MW as hosts for nematodes, they were exposed to various host-stressor regimes, whereas the nematodes, in contrast, were exposed to infectivity-enhancing additives. Three treatments, plus a control treatment, were compared: hot-water treatment of MW; infective juvenile (IJ) stimulation by $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$; and a combination of hot-water MW treatment with IJ stimulation by $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$. Hot-water treatment of insect larvae was selected above that of dry-heat treatment for the study, even though dry-heat treatment led to more consistent results in terms of IJ yield production in the study by Brown et al. (2006). The reason for choosing the hot-water treatment was that, under a small-scale producer's conditions, where the funds that are available for capital investment are limited, hot-water treatment would be a more practical and cost-efficient method to implement, provided that acceptable levels of infection of insect larvae can be achieved.

Pre-inoculation heat treatments of mealworms proved to be unsuccessful in increasing EPN infection of MW. Reduced infection levels, when using *H. bacteriophora*, occurred after MW were exposed to 70°C water, compared to that which was used in the control. Only 12% and 13% infection was obtained with 70°C water treatment and the 70°C water treatment combined with the $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$ -treated IJs respectively, compared to the 48% that was obtained with the

control treatment. Despite the mortality being 100% for both hot-water treatments, infection levels remained low. The same trend was observed with *H. zealandica*, with which the infection was 21% and 9% for treatments 70°C and 70°C + $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$, respectively. A study undertaken by Brown et al. (2006) demonstrated that exposure of MW larvae to 65°C and 70°C water increased infection by *H. bacteriophora* compared to that which was attained with the control. Exposing the insect larvae to high temperatures compromises effective functioning of insect muscles responsible for closing bodily orifices, thus keeping the spiracles and anus open for periods long enough to facilitate nematode penetration. Furthermore, an anti-microbial protein, tenecin 1, which is found in MW larvae, and which has a negative influence on the bacterial symbiont of *H. bacteriophora*, is denatured when exposed to high temperatures, thus overcoming such a defence mechanism and increasing the susceptibility of MW (Brown et al. 2006). Despite the effect that hot-water treatment had on MW larval physiology, infection levels were not increased in the current study. A possible reason for the lack of such an increase can be due to the longer period (five seconds) of MW immersion in 70°C water in the current study, compared to the one-second immersion of MW in the study by Brown et al. (2006). The longer period of immersion in hot water could have affected the nutritional value of MW larvae, making them an unattractive, suboptimal substrate for nematode penetration and proliferation.

As opposed to findings recorded in prior studies, in which manganese ions considerably increased the infectivity of *H. bacteriophora* against insect hosts (Jaworska et al. 1997; Brown et al. 2006), similar results were not obtained in the current study. *Heterorhabditis zealandica* IJ infectivity was slightly increased under application of a treatment with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$, and was the most effective treatment in increasing MW infectivity, causing a 1% higher infection rate (81%) compared to that which was attained with the control (80%). Said treatment did, however, not have similar effects on *H. bacteriophora*, where infection levels were 1% lower than with the control, at 47%. According to Jaworska et al. (1999) and Brown et al. (2006), manganese and magnesium ions have a protective effect on nematodes, by pairing with other metal ions in a solution that is toxic to nematodes, that reduces nematode mortality and that also increasing its virulence towards such hosts as WML, MW and *Sitona lineatus* (L.) weevils. A possible explanation for the lack of infectivity enhancement obtained in the current study by means of treating IJs with $\text{Mn}^{2+}\text{SO}_4\cdot\text{H}_2\text{O}$, is that the contact period between the ions and nematodes was too short. At no stage were the nematodes in contact with the ions for longer than one h at a time, whereas exposure lasted for up to 96 h in the experiments conducted by Jaworska et al. (1997) and Brown et al. (2006). In addition to the time factor, it has been shown that *S. carpocapsae* is much more predisposed to the positive effect that such ions have on nematode pathogenicity, compared to *H. bacteriophora* (Jaworska et al. 1997).

Based on the results obtained in the current study, significant differences in the effectiveness among inoculating treatments investigated were observed. Pipetting was the treatment that caused the highest levels of infections in most of the nematode–host combinations. However, inoculation by immersion was selected as the method of choice for both WML and MW, due to its time-efficiency and ability to produce acceptable levels of infection in the case of WML. The lower levels of infection obtained with MW may, however, be acceptable in some instances, if it proves to be a cost-effective option when compared to the use of WML. Major improvement in nematode infection rates, using chemical and physical stressors in previous studies, did not resonate in the current study. However, streamlining the protocol followed in the study by optimising such parameters as the exposure time of both nematodes to manganese ions and hosts to hot water could amplify the positive effect that such stressors have on infection rates.

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Chapter 5

Formulation of *Heterorhabditis zealandica* and evaluation of a quality control measure

Abstract

A comparative study was conducted to determine the storage stability of *Heterorhabditis zealandica* in modified Pesta granules and coconut fibres compared to the control, which represented the suspension of nematodes in distilled water. Nematode survival within each formulation was determined after 24 h and thereafter at 7, 14 and 21 day intervals. Formulations were stored at 4°C. Storage stability in both formulations decreased during the storage period. Only 9.79% *H. zealandica* infective juveniles (IJs) survived 21 days in Pesta granules, and 2.25% in coconut fibres, respectively. The percentage IJ survival in the control for both Pesta granules and coconut fibres was much higher, at 79.79% and 100%+, respectively. The Pesta granule and coconut fibre formulation proved to be unable to maintain nematode survival. The quality control measure implemented to determine the virulence of formulated nematodes, using the percentage mortality of mealworms as an indicator, was not effective. Unreliable results were obtained using the method concerned. As a result, the virulence of formulated nematodes could not be determined. An adapted or alternative storage formulation and quality control measures should be investigated for *H. zealandica*.

Introduction

Entomopathogenic nematodes (EPNs) are insect parasites with the ability to control insect pests and are considered to be an effective alternative to chemical insecticides within an integrated pest management programme (Gaugler and Kaya 1990; Kaya and Gaugler 1993; Grewal 2002). EPNs from the families Steinernematidae and Heterorhabditidae are produced on a commercial scale as bio-insecticides and have been used for the biocontrol of numerous insect pests (Peters and Ehlers 1994; Gaugler and Han 2002; Georgis et al. 2006; Caamano et al. 2008). Two of the most important aspects for a biological control agent to be successful is the ability to be mass-produced and formulated. Freshly harvested nematodes at high concentrations will perish if stored in water, mainly because of a lack of oxygen. Therefore, nematodes should be formulated to keep them stable during storage by limiting mortality and maintaining infectivity (Silver et al. 1995; Strauch et al. 2000). Apart from preserving nematodes, formulations assist in the

handling, transport, application and enhancement in field efficacy of the nematodes (Jones and Burges 1998; Grewal and Peters 2005). Despite the successes obtained using EPNs as biocontrol agents, development of formulations that extend shelf-life as long as a year, while ensuring delivery of high-quality nematodes, have not yet been realised (Gaugler and Han 2002; Chen and Glazer 2005; Georgis et al. 2006).

The unique challenge of maintaining biological activity in formulations containing nematodes is that these living organisms demand sufficient levels of oxygen and moisture (Georgis 1990; Georgis and Kaya 1998; Sharma et al. 2011). When developing or selecting a formulation, the requirements specified should be taken into account. To determine the requirements of specific nematode species, the physiological chemistry, ecology and behaviour of the species need to be understood (Georgis 1990; Georgis and Kaya 1998). The absence of a stable and effective formulation, capable of maintaining nematode quality for a few months, might be due to the complexity of the combination of factors that needs to be known and taken into consideration during formulation development. In addition to that, secrecy of information on the topic in the commercial industry limits chances for improvement of formulations.

Two distinct approaches to formulating nematodes have been used, from which a variety of formulations have branched. Firstly, the use of such inert carriers as sponge (Bedding 1984), and secondly, the use of inert carriers containing such additional functional ingredients as adjuvants and antimicrobial agents. Small-scale nematode producers generally use low-technology and -cost formulations, such as sponge, or store nematodes in aerated water under refrigeration conditions, under which conditions it can be kept for up to several months (Shapiro-Ilan and Gaugler 2002). Although inert carriers are less expensive and assist in the transport and handling of nematodes, nematodes can move around freely and, in some cases, migrate from the formulation. The high rate of activity of nematodes in such formulations leads to the depletion of stored energy reserves, which, in turn, leads to increased sensitivity to extreme temperatures and reduced IJ viability and pathogenicity (Womersley 1990a, b). Furthermore, nematodes that migrate from the formulation, dry out and die. To overcome such problems, nematodes are increasingly being formulated into non- or semi-liquid substrates, such as absorbent clays and water-dispersible granules (WDG), which physically trap nematodes, contain metabolic inhibitors and induce partial anhydrobiosis (Grewal 2002; Grewal and Peters 2005). Anhydrobiosis is a reversible, physiologically arrested, state of dormancy resulting from a total loss of body water (Grewal 2000), which is more common in plant-parasitic nematodes of the family Anguinidae. However, EPNs in the families Heterorhabditidae and Steinernematidae can only enter a state of partial anhydrobiosis (Jagdale and Grewal 2007). The development and modification of granular formulations by Capinera and Hibbard (1987), Connick et al. (1993) and Silver et al. (1995) contributed significantly to the advancement of formulation development. *Steinernema* IJs survived for up to six months in an anhydrobiotic state at 4-25°C in a WDG. The nematodes were encased within

the granule, consisting mainly of various types of clays and starches (Grewal 2002; Shapiro-Ilan and Gaugler 2002). The WDG formulation was the first formulation allowing Steinernematids to survive for up to six months at 25°C. Unfortunately, the shelf-life of heterorhabditids is much shorter than that of steinernematids, due to higher sensitivity to desiccation and temperature, different foraging behaviour and a faster rate of lipid use (Georgis et al. 1995; Bilgrami and Gaugler 2007). The few commercially produced heterorhabditid formulations also require constant refrigeration (4-6°C) to ensure nematode storage stability (Selvan et al. 1993; Georgis and Kaya 1998).

Due to the extensive advances that had been made in attempting to gain a better understanding of different nematode species requirements, formulations can be developed to conform, to an extent, to specific requirements (Strauch et al. 2000). However, variation in nematode stability during long-term storage still occurs within such formulations. Accordingly, effective quality control (QC) measures should be set in place to ensure nematode stability in formulations, as well as during the entire production process (Boff et al. 2000). QC is defined by Grewal and Peters (2005) as “a system of maintaining standards in manufactured products by testing a sample of the output against the specification”. Nematode virulence is one of the most important indicators of nematode quality, and several measures exist to determine it (Grewal 2002; Grewal and Peters 2005). With the most effective and practical QC measures in place, high-quality endemic nematodes can be produced on a small scale for research laboratories, niche markets and organic grower cooperatives.

In the current study, two nematode formulations of a local endemic South African EPN were compared for survival and virulence. The objective was to determine which formulation ensured optimal survival of *H. zealandica* without compromising virulence.

Material and methods

Source and storage of nematodes and host insects

The *Heterorhabditis zealandica* Poinar 1990 strain SF41 (Genbank accession number FJ455843) used in this study was taken from the Stellenbosch University nematode collection and isolated during a survey for endemic EPNs (Malan et al. 2006). IJs for inoculum were produced *in vivo*, based on the White trap method described by White (1927) and Kaya and Stock (1997). Laboratory-reared (Chapter 2) last-instar wax moth larvae (*Galleria mellonella* L.) (WML) were used as hosts for nematode production and were inoculated with 200 IJs / larva. Nematodes were harvested within the first three days of emergence, and then transferred to 1-L aerated glass cylinders at 14°C, in which they were stored for up to

three days, before being formulated. Last-instar mealworm larvae (*Tenebrio molitor* L.) (MW), used in the QC experiments, were obtained from a laboratory culture reared on wheat bran in a temperature-regulated room at 25°C (Chapter 2).

Preparation of nematode storage in Pesta granule

The Pesta granule formulation consisted of 864 g semolina, 108 g bentonite and 108 g coconut fibres ground individually, sieved through a 250-µm aperture screen and then mixed together. IJs harvested from 250 WML were counted according to the method followed by Glazer and Lewis (2000), and $\pm 9\,520\,000$ nematodes were concentrated using a 10-µm sieve. The nematodes were then washed from the sieve surface with 56 ml of 0.1% formaldehyde, into a 150-ml glass cylinder. Every 50 µl thus contained 8500 nematodes. A plastic pipe connected to a fish-tank pump was immediately inserted into the cylinder to ensure the survival of nematodes by means of the aeration of the nematode/formaldehyde suspension throughout the preparation of the WDG. Nematodes were manually applied to the dry Pesta powder in drops of 200 ml, using an Eppendorf multi-pipette. Each drop contained $\pm 34\,000$ nematodes. As soon as the drops containing the nematodes came into contact with the powder, the container with the powder was gently swirled, in order to encapsulate the nematodes, until a granule was formed with an approximate diameter of 2 cm. Five granules, encapsulating $\pm 170\,000$ nematodes, were placed in a plastic sachet and sealed. A total of 56 sachets were filled for each trial that was conducted on different test dates, and stored at 4°C.

Preparation of nematode storage in coconut fibres

Heterorhabditis zealandica, harvested from 250 WML, were concentrated to form a solution containing $\pm 170\,000$ nematodes / ml distilled water. One ml of nematode suspension, together with 4 ml of a 0.1% formaldehyde, was added to 5 g dried coconut fibre and manually mixed. The 5 g coconut fibre formulation was then sealed in plastic sachets and immediately placed at a temperature of 4°C. A total of 56 sachets were formulated for each of the two trials that were conducted on different dates.

Preparation of nematode storage in water

A total of $\pm 9\,520\,000$ nematodes from each of the respective inoculum batches prepared for the two formulations, were retained on the surface of a 10- μm sieve and decanted in a 5-L cylinder, to which 2 380-ml tap water was added. A volume of 42.5 ml of this nematode suspension was added to ventilated 175- cm^2 culture flasks and stored at 14°C in the dark. Each culture flask thus contained $\pm 170\,000$ IJs. Flasks were shaken once every week for the duration of the study, to facilitate aeration. Storing nematodes in water was used as control for both the Pesta and coconut fibre formulations. Nematodes produced from the same batch WML were used for the control and corresponding formulation. Therefore, each formulation trial consisted of 56 culture flasks used as the Pesta formulation control and 56 flasks used as control for the coconut fibre formulation. The same procedure was followed when the trial was repeated on a different test date.

Survival and infectivity quality control assessment (QC)

The nematode survival quality control assessment involved counting the number of nematodes the first day after formulation and thereafter on days 7, 14 and 21, to ascertain their survival rate. The infectivity assessment involved determining the infectivity of formulated nematodes by assessing MW mortality after inoculation with nematodes obtained from a formulation for each time period concerned. The infectivity quality control assessment was conducted directly after the survival quality control assessment. In total, four QC assessments were conducted 24 h after formulation and thereafter on days 7, 14 and 21.

For the QC (survival and infectivity) in the Pesta formulation, the content of 14 sachets, containing $\pm 2\,380\,000$ IJs, was poured into 238-ml water in a 500-ml glass beaker. The suspension was vigorously stirred for one minute and kept agitated by bubbling air, until granules had disintegrated. For QC of the fibre formulation, nematodes were extracted from the fibres by using a modified version of the Baermann funnel technique (Staniland 1954; Viglierchio and Schmitt 1983). Each week, the content of 14 sachets containing 2 380 000 IJs, were placed in round sieves with a mesh screen base of 45 μm . The mesh size would allow nematodes to pass through into the surrounding water, while retaining fibres. The sieves containing the coconut fibre formulation were then placed in the middle of a metal dish and tap water was poured over the formulation, until the sieve was surrounded by a pool of water. After leaving sieves for 24 h at 25°C in the metal dish, the surrounding water containing nematodes was decanted into a 5-L plastic container. Nematodes present in retained fibres in sieves, were washed out by applying water at high pressure to the fibres for two minutes. The water containing nematodes was also collected in the 5-L plastic container. The total volume of water collected, was reduced to 238 ml, by pouring it through a 10- μm sieve, while in the process retaining nematodes. The

nematodes were then decanted with 238-ml water from the sieve into a 500-ml glass container and kept aerated with a fish tank pump, until QC assessment was completed. For QC of the control (nematodes stored in water) for both formulations respectively, the content of 14 culture flasks, containing $\pm 2\,380\,000$ IJs, was poured into a 500-ml glass beaker and reduced to 238 ml when the nematodes settled to the bottom of the beaker. Air was bubbled through a tube connected to a fish-tank pump into the nematode suspension, in order to ensure the survival of nematodes during the assessment process. Applying to all formulations including their controls, three 100- μ l samples of the 238-ml nematode suspension were taken with a micro-pipette and injected into three separate test tubes. To dilute the suspensions, 4.9-ml tap water was added with a micro-pipette to each test tube and the test tubes were shaken to ensure sufficient mixing of fluids. Immediately after the test tubes were shaken, five 100- μ l samples were taken from each test tube and placed on a clean Petri dish. After having placed five droplets from each test tube into separate Petri dishes, the number of living nematodes within each droplet was counted. Living nematodes were distinguished from dead nematodes according to the criteria by Peters (2004). Further calculations to determine the number of living nematodes in 238 ml were conducted according to the COST 850 quality assessment protocol developed by Peters (2004).

Testing for infectivity of nematodes remained the same for all formulations tested. For each trial, 1300 g quartz-sand (0.1-0.4-mm grain size) was dried and water content adjusted to 10% by addition of 130-g distilled water. The water and sand was mixed thoroughly and 200 g was placed in each of six plastic containers (10 \times 10 \times 2 cm). Nematode suspension prepared in the first procedure for counting of nematodes, was further diluted into six different doses, according to the protocol developed by Peters (2004). Nematodes were applied (1 ml) to the quartz-sand in the middle of the plastic container, in the following dilutions: 0, 200, 400, 800, 1200 and 2400 nematodes/ml. After nematode application, 40 last-instar MW larvae were added to each container. Containers were then closed with a lid containing 20 needlepin holes and incubated at 25°C in the dark. MW mortality was determined in each container after seven days. A MW was deemed dead if no movement was observed when prodded with a pair of tweezers. QC trials for both formulations and its corresponding controls were repeated on a different test date.

Data analysis

Data was subjected to analysis of variance (ANOVA) using Statistica version 10 (StatSoft Inc. 2011) data analysis software system. Results obtained from nematode counting for QC were analysed using a one-way ANOVA. The results obtained were analysed after the two trials of each procedure had been pooled. Significant differences in treatments were determined with post-hoc comparisons of means, using the least-significant-difference (LSD) test (Jones 1984). A factorial ANOVA was used to analyse the results obtained from the QC procedure measuring nematode

infectivity. Prior to analysis, MW mortality was corrected for the aforementioned procedure using Abbott's (1925) formula to compensate for death of insects through natural means.

Results

Nematode survival in formulations

When counted the day after formulation, the number of IJs that survived were 1 134 467, 1 491 467, 706 067, and 1277 267, respectively in Pesta granules, Pesta control, coconut fibres and coconut control (Table 1). The initial total of nematodes formulated within 14 units (sachets or culture flasks) for every week was $\pm 2\,380\,000$ IJs. The first count was used as baseline in determining nematode survival. IJ survival after 21 days in Pesta granules and coconut fibres was, respectively, 9.79% and 2.25%. In the Pesta and coconut fibre control, respectively 79.79% and 100%+ of IJs were still alive after 21 days. The only formulation in which IJ survival continuously decreased at a constant rate over a period of 21 days, was in coconut fibres. The percentage IJs that survived in this formulation decreased from 25.84% after 7 days in the formulation, to 2.25% after 21 days. Approximately 50% of the IJs were lost in the Pesta formulation during the formulation and retrieval process. In the coconut fibre formulation, approximately 70% of the IJs were lost.

Table 1 The influence of the time of storage on the mean \pm SE (standard error of the mean) number and percentage survival of *H. zealandica* IJs stored at 4°C in Pesta granules, coconut fibres and distilled water (Pesta and coconut control). The SE for all values is 515 899.

Formulation	Storage time							
	24 h		7 days		14 days		21 days	
Pesta granules	1 134 467	100%	1 681 867	100%+	277 667	24.48%	111 067	9.79%
Control - Pesta	1 491 467	100%	1 245 533	83.51%	1 618 400	100%+	1 190 000	79.79%
Coconut fibres	706 067	100%	182 467	25.84%	15 867	2.25%	15 867	2.25%
Control - fibres	1 277 267	100%	1 483 533	100%+	1 396 267	100%+	2 181 667	100%+

Nematode infectivity after formulation

No significant interaction occurred between the two trials of this procedure. Therefore, results from each trial were pooled. Significant differences occurred among combined mortality obtained in all four formulations with different nematode dosages ($F_{(5, 178)} = 9.7527$, $p < 0.001$). An increase in MW mortality was observed, with an increase in

nematode dosage applied. Inoculating MW larvae with 200 nematodes led to mortality of $12.35\% \pm 3.44\%$ (mean \pm SE), followed by $19.85\% \pm 4.32\%$, $35.50\% \pm 5.30\%$, $35.09\% \pm 5.73\%$ and $46.30\% \pm 6.58\%$ when inoculating with 400, 800, 1200 and 2400 nematodes respectively. However, the mortality rate obtained of MW larvae when no nematodes were applied was found to be high. The application of no nematodes to units containing MW larvae led to mortality of $53.44\% \pm 3.71\%$ (Fig. 1).

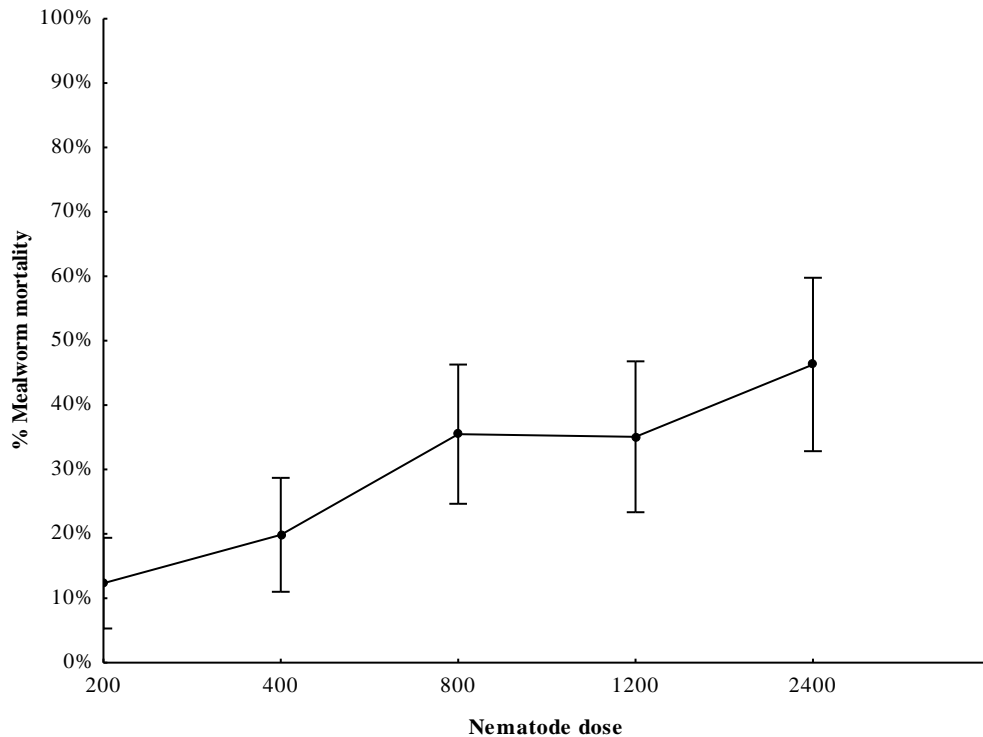


Fig. 1 The Abbott's corrected percentage mortality of mealworm larvae (mean \pm SE) at various nematode dosages. A 95% confidence interval was used (one-way ANOVA; $F_{(5, 178)} = 9.753$; $p < 0.001$).

Discussion and conclusion

In vivo producers tend to use formulations that are cost-efficient and that require unsophisticated technology, such as storage of nematodes in water or sponge. Despite being an effective means of nematode storage when low numbers are produced, the method is not practical when higher yields are needed for field applications (Shapiro-Ilan and Gaugler 2002). In an *ad hoc* trial leading up to the experimental trials performed in the current study, five other formulations were initially considered, beside coconut fibres and Pesta granules. These five formulations included: infected WML coated with vermiculite and bentonite respectively (Koppenhöfer et al. 1997; Shapiro-Ilan et al. 2001; Del Valle et al. 2009); activated charcoal; vermiculite and kaolin as clay component in Pesta powder formulations; and peat fibres. Formulations were stored at 4°C and 25°C for seven days. It was concluded that the formulations were not acceptable methods for storage of *H. zealandica*. Formulations were impractical, expensive, time-consuming to prepare, prone to contamination, or no nematodes survived in the formulation at the specific temperature after seven days. Pesta granules and coconut fibres performed best in terms of the above-mentioned criteria and were, accordingly, selected for the continuation of experimental trials. A storage temperature of 4°C was selected, since higher nematode survival was obtained in the *ad hoc* trial in formulations stored at this temperature, compared to those that were stored at 25°C. Higher nematode survival at lower storage temperatures, obtained in studies conducted by Klingler (1990), Connick et al. (1993); Boff et al. (2000), and Strauch et al. (2000), supports the finding. In laboratory conditions, nematodes are generally stored in water at low temperatures. Therefore, this method was used as a control for the coconut fibre and Pesta granule formulations.

The Pesta granule formulation used in this study is a modification and simplified version of the water-dispersable granule (WDG) developed by Silver et al. (1995). The exact composition of this WDG is unknown, due to patenting rights. However, ingredients that could have been used in combination or alone to develop the granules were mentioned and included the following: silica; various types of clay; starch; diatomaceous earth; and cellulose. The simplified WDG used in this study consisted of semolina, bentonite, coconut fibres and 0.1% formaldehyde. Semolina is used as a binder in nematode formulations to ensure that granules remain intact (Connick et al. 1993). Bentonite is an inorganic silicate and is the diluents ingredient in this formulation. The function of diluents is to dilute the nematodes to a concentration that would be able to control an insect pest when the formulation containing nematodes is applied to the target insect. Even though bentonite is inert, it is still chemically active and possesses water-retaining qualities, which, in turn, can be beneficial to EPNs that require a certain degree of water activity during storage. Apart from their water-retaining capabilities, clays physically protect nematodes from UV light, have a high buffering capacity, reduce pH fluctuations within formulations, and facilitate aeration (Ward 1984). Furthermore, bentonite is widely available and is

relatively inexpensive. Grounded peat fibres were added to the formulation, as they have water-retaining capabilities and promote disintegration of granules in water, thus acting as a surfactant (Connick et al. 1993). Formaldehyde was added to serve as an antimicrobial embodiment, restricting unwanted microbial growth and contamination, as WDG have been shown to be particularly prone to microbial contamination (Yukawa and Pitt 1985; Chen and Glazer 2005; Grewal and Peters 2005).

The number of nematodes that were alive the day after being formulated was much less than the initial concentrations. The main reason for the apparent reduction in nematode survival is the loss of nematodes during the formulation process, and not the result of storage time. Even though 2 380 000 IJs were initially formulated into the 14 sachets dissolved in water each week to be counted together with its control (nematodes in water), the number eventually formulated was much lower, due to possible spillage or death of nematodes during handling and formulation. Nematode producers often over-pack commercially formulated nematode products, thereby compensating for nematode loss during formulation and handling. As a result, the number of nematodes counted 24 h after being formulated was used as the baseline to determine nematode survival. The difference in the perceived number of nematodes formulated and the actual number being formulated gives a good indication as to how many nematodes are lost during the formulation process.

Great variation in *H. zealandica* IJ survival occurred from week to week. The number of nematodes seven days after formulation to 14 days after formulation in Pesta granules showed no decrease and can be regarded as the same. Poor extraction of nematodes from the formulation when dissolved in water seven days after being formulated and loss of nematodes during formulation could possibly have been due to the apparent lower levels of nematode survival. Addition of surfactants in higher quantities when formulating the granules may increase solubility of the product and consequent nematode release. However, even though extraction of nematodes from the formulation proved to be challenging, the number of nematodes that died in the formulation increased substantially over time. The 9.79% of nematodes that were alive after 21 days in the Pesta formulation is much lower compared to the 2-3 months that *H. zealandica* survived in a wettable powder at 2-10°C (Grewal and Peters 2005). As a result, this Pesta granule formulation would not be an effective means of storage for the local *H. zealandica* isolate used in this study. Despite the beneficial properties of ingredients in the Pesta granule formulation, a rapid desiccation rate might have inhibited the nematodes to adjust properly to the increased osmolarity (Strauch et al. 2000). The ingredients in this formulation act as absorbents and aid in inducing partial anhydrobiosis (Grewal 2000). Inducing partial anhydrobiosis in nematodes is said to be the best way to increase its shelf-life (Grewal 2000). Metabolic rate of nematodes is decreased in this state, and they tend to be more tolerant to extreme temperatures (Georgis and Kaya 1998; Grewal and Peters 2005). However, a gradual process of desiccation by reduction in water activity is key to initiating partial anhydrobiosis in nematodes.

Rapid desiccation would lead to nematode death, and the low survival rates obtained in the current study might partially be due to such a factor (Simons and Poinar 1973). The drying rate of the granules should thus be optimised to prolong shelf-life. Additionally, *Heterorhabditidae* are known to be more sensitive to such abiotic factors as temperature and humidity, compared to *Steinernematidae* (Georgis et al. 1995; Bilgrami and Gaugler 2007). Since the most commonly formulated nematode species are *Steinernema*, the long shelf-life obtained with certain formulations is based on the use of these species and not on the use of *Heterorhabditis* (Georgis and Kaya 1998; Boff et al. 2000).

Very low percentage survival rates of *H. zealandica* were obtained throughout the current study, using the fibre formulation. Survival rate decreased from 25.84% after seven days to 2.25% after 21 days (Table 1). With the Pesta granule formulation, nematodes were encapsulated and mobility limited as opposed to the fibre formulation, where nematodes were free to move around. Due to the structural difference between the two formulations, nematodes in the fibre formulation could possibly have died due to desiccation or due to rapid use of energy reserves, leading to the higher numbers of dead nematodes in the fibre formulation compared to the Pesta granule formulation (Georgis et al. 1995). Conversely, weekly variation in nematode survival rate that occurred in controls of both Pesta granules and coconut fibres could be due to batch variations. According to Griffin (1996), *Heterorhabditis* is known for batch variation. Decanting nematodes from one container to another during the concentration process could also have led to varied numbers of nematodes being placed in the culture flasks concerned.

Standard procedures to evaluate nematode quality do not exist, but field applications are generally used as a measure of nematode quality (Caamano et al. 2008). The method is, however, not always practical to implement, especially in the case of small-scale nematode production, where inoculum is limited. A quality control measure developed by Peters (2004) was applied to quantify the effect that storage time had on the virulence of formulated nematodes. From the results obtained, it was impossible to determine the nematode virulence, due to the overall mortality rate of MW being the highest for the control (53%), even though no nematodes were applied to the larvae. The conclusion that could be drawn from the above is that the MW larvae died of causes other than nematode infection. It was noted that the quartz-sand used in each experimental unit (plastic container), was very moist, which creates a suitable environment for unwanted microbial growth. MW larvae also prefer dry conditions to wet conditions. A suboptimal nematode–host combination of MW and local *H. zealandica* isolates used could further have contributed to reduced infection levels and skewed results. Modifying the existing technique, by replacing MW hosts with hosts that are more susceptible to the specific nematode isolate used, might improve its effectiveness as a quality control measure. In contrast, the quality control technique used could be substituted with a different technique that is specifically suited to the endemic isolates that were used.

In conclusion, none of the EPN formulations tested performed better than did the control. Due to the shortage of information on the compatibility of *H. zealandica* with formulation ingredients, further research should focus on determining the effect that various formulation ingredients have on *H. zealandica*. Basic ingredients were used in the formulations tested and the addition of certain additives, such as humectants, preservatives and anti-caking agents, might increase its effectiveness. It should be noted that formulations still need to be cost-efficient after the addition of these additives. The quality control measure used was not effective in determining the virulence of endemic nematode isolates. To ensure the production and storage of a high-quality product, the protocol of the method can either be modified, or an alternative method should be investigated.

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Chapter 6

General conclusion

The overall aim of the project covered in the current thesis was to contribute towards the effective small-scale production of endemic entomopathogenic nematode (EPN) species through the enhancement and streamlining of existing practices for *in vivo* nematode production. The first step in the *in vivo* production process is choosing a susceptible host. Therefore, comparative studies based on host suitability and cost-efficiency were conducted using several hosts and two endemic nematode species, *Heterorhabditis zealandica* and *H. bacteriophora*. The next step focused on in the *in vivo* production process was to improve inoculation methods, using both *Galleria mellonella* (L.) and *Tenebrio molitor* (L.) as insect hosts. Furthermore, methods to increase infectivity of *H. zealandica* and *H. bacteriophora* and, consequently, to ensure higher rates of latent host infections, was investigated. Lastly, various nematode formulations were compared, making use of a standard quality control measure, with the aim of selecting a superior formulation, according to highest nematode survival rate and best maintenance of nematode virulence.

Cost-effective culturing of *Galleria mellonella* L. (greater wax moth) and *Tenebrio molitor* L. (yellow mealworm) larvae, through selection of respective superior diets

Wax moth larvae (WML) and mealworms (MW) proved to be the best-suited hosts to use for *in vivo* production of local *H. bacteriophora* and *H. zealandica* isolates, partly due to the large numbers of nematodes they produced per g host, in comparison to false codling moth (*Thaumotobia leucotreta*)(FCM) and the codling moth (*Cydia pomonella*) (CM). WML yielded 1 459 205 and 1 898 512 infective juveniles (IJs) per g host of *H. zealandica* and *H. bacteriophora*, respectively. MW larvae produced less IJs compared to the number produced by WML, but significantly more compared to that which was produced by CM and FCM. The number of *H. zealandica* and *H. bacteriophora* IJs respectively produced by MW were 836 690 and 414 566 per g host. Another reason for WML and MW being chosen as nematode hosts was the ease of rearing such hosts on artificial diets in the laboratory. One superior diet for each of the insect hosts was selected, based on the interaction between cost-efficiency of the diet and average weight accumulation of hosts reared on the specific diet. The diets concerned were Diet 1 (wheat flour, wheat bran, milk powder, brewer's yeast, wax powder, honey and glycerol) for WML and Diet 5 (wheat bran only) for MW larvae.

Improving nematode infectivity by selection of an effective inoculation method and manipulation methods for both nematode and host

Three different inoculating methods were compared using *H. zealandica* and *H. bacteriophora*, respectively, with the aim of selecting one practical method, which provided the highest infection rate of WML and MW. Pipetting produced the highest rate of infection for both nematode species and insect hosts. Immersing hosts in a nematode suspension proved to be the most practical and time-efficient method of inoculation, especially in the case of WML, where high infection rates were obtained. Although lower infection rates were obtained, compared to those that were obtained with WML when MW were immersed into a nematode suspension, immersion was still found to be the most practical inoculation technique to use for small-scale production. The results obtained from the current study not only indicated which inoculation technique proved to be the most effective, but also gave an indication of the practicality of using a certain host, based on its susceptibility to the endemic nematode species used. To further improve infection rates during the *in vivo* production process, the effect of three treatments on infectivity, by imposing physical and chemical stress upon host and nematode, was investigated. Despite the positive effect that the stressors concerned were found to have on infectivity enhancement in other studies, the same effect was not observed in the present study. Physical and chemical stressors had an insignificant influence on the infectivity of nematodes. Future research on infectivity enhancement of the endemic nematode species, *H. zealandica* and *H. bacteriophora*, used in the study should be aimed at optimising the experimental protocol.

Comparing various customised nematode formulations and selecting the most promising formulation according to results obtained, using a specific nematode quality protocol

Heterorhabditis zealandica proved to be very sensitive to storage conditions using the formulations tested. IJ survival in Pesta granules and coconut fibers at 4°C decreased substantially after 21 days. The lowest survival rate was obtained using coconut fibers, with only 2.25% survival being observed after 21 days in storage. Survival in Pesta granules was 9.79% after the same storage period. Higher survival rates were obtained for the control treatments of both formulations where nematodes were stored in a suspension of water in culture flasks at 14°C. Nematode survival was 100%+ and 79.79% after 21 days in storage for the coconut fiber control and Pesta control, respectively. The deduction is that, as neither of the formulations used was capable of maintaining nematode activity, neither would be an effective means of

storage. Even though the survival of IJs in water (control) was higher compared to what was achieved in the formulations tested, water is not an effective method of nematode storage for extended periods of time. Furthermore, the impracticality of such a method of storage excludes it from being used in conditions where nematodes are produced on a larger scale. The quality control protocol, developed by Peters (2004) and used in this study, was not effective in determining the rate of MW mortality caused by formulated *H. zealandica* IJs at different doses. Therefore, the virulence of IJs could not be successfully measured. Future research should be directed at either customising this quality control method for South African nematode strains or at implementing alternative quality control measures for the formulation of endemic nematodes.

Research conducted during the current study contributes to the optimisation of selected steps in the *in vivo* mass rearing of both endemic strains of *H. zealandica* and *H. bacteriophora*, which was not previously available. The continuous focus on cost-effectiveness in the steps studied makes the findings relevant and applicable to parties wanting to produce nematodes on a small scale. In such instances as the formulation development trials, where formulations failed to maintain nematode infectivity and survival for a sufficient period of time to be considered effective, the sensitivity and different reaction of local *Heterorhabditis* isolates to formulations and treatments usually used for Steinematids was once again highlighted. In such instances, suggestions are given on how possibly to solve, or improve, the problem. It is believed that in the trials specifically conducted to improve nematode infection rates through physical and chemical stress, minor protocol alterations could change the outcome of such treatments from having a mild effect on the nematode infection rates to significantly increasing the infection rates concerned. Furthermore, certain areas in the production process have been pinpointed where the lack of information available to ensure the efficient production of these nematodes is evident, and future research in such areas is suggested.